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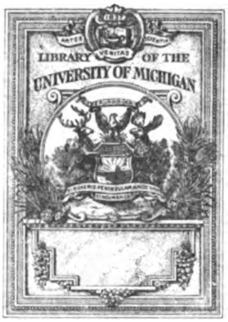
Biological Papers ...

Bertram Garner Smith



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COLLECTION AND PREPARATION OF MATERIAL FOR CLASSES IN ELEMENTARY ZOÖLOGY.

BERTRAM G. SMITH.

During the past year the writer has frequently received inquiries concerning methods of collecting and preparing zoölogical material for class use. The purpose of the present article is to give some of the methods used in the Department of Zoölogy of the University of Michigan in supplying a class of over a hundred students with laboratory material for a first course in elementary invertebrate zoölogy. A few organisms are included that are not ordinarily studied in a beginners' course, as some of them may be found useful for demonstrations, or for supplementary work. On the other hand, no attempt is made to make the list comprise all the forms adapted to a first year's course; for many of these, directions for collecting would be superfluous.

1. *Amœba.*[1]— Collect the water plant Ceratophyllum, which will be found growing in the quiet water of ponds and rivers, in the same habitats with water lilies, Elodea, etc. Gather the Ceratophyllum in considerable quantities, packing it in pails

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[1] H. S. Jennings. "Methods of Cultivating Amœba and Other Protozoa for Class Use." *Journ. of Applied Micr.*, vol. 6, no. 7, p. 2406.

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with enough water to cover. It is best to get material from several localities. If Ceratophyllum cannot be found, Elodea and various other plants may be used, but the results are less satisfactory.

Bring the material to the laboratory and there set up cultures as follows. Use "bacteria dishes" — shallow circular glass dishes, about 9 inches in diameter and 3 inches deep. Pack the material in these *firmly*, crushing it down. This will prevent growth of the plant, and favor fermentation. Pack to within an inch of the top of the dish, and add just enough water to cover the material. Cover the dishes with glass plates and put them in various warm and well lighted places about the laboratory. Each culture should be labeled with the *place* where the material was obtained, and the *date* when the culture was set up. Later, as organisms appear which are to be studied, their *names* should be added to the labels. *Label the dishes, not the covers.* If water evaporates so as to expose the plant material, enough should be added from time to time to make good the loss.

The first cultures should be started about *three weeks* before the Amœbæ are needed. A half dozen cultures should be started at this time and several new ones added every two or three days thereafter, until the class is nearly through studying Amœba. The time required for the cultures to mature varies; a single culture may last but a few days, and not all are successful — hence the necessity for frequent collections and many cultures.

The first indication of Amœba is a brown or a white scum appearing on the surface of the water. This consists mainly of bacteria, and the Amœbæ come to the surface to feed on them. Take up a bit of the scum with forceps, mount in a drop of water taken from the surface with a pipette, tease out the scum thoroughly with needles, and examine with a microscope. Amœbæ, if present, will usually be found among or near the particles of scum. Also, remove a bit of the plant material and scrape the slime from it. This will often contain more Amœbæ than the scum at the surface of the water. The specimens are small at first, but in a few days they become larger.

While the Amœbæ usually disappear from such a culture in a short time, occasionally they persist for a week or more, growing larger. In some cultures they seem to encyst or disappear almost entirely, and later reappear in considerable numbers. Hence old cultures should be watched, and retained until they become overgrown with fungi and hence worthless.

The advantages of the Ceratophyllum method, as described and used by Jennings, are that it gives Amœbæ in abundance, at the time desired, with almost absolute certainty. But the Amœbæ obtained by this method are seldom large; their development is often interfered with and they usually give place to other organisms before attaining maturity. Moreover, the presence of an excessive amount of bacteria and other organisms often obscures the Amœbæ, and makes it difficult to find and study them. To overcome these difficulties, the writer, aided by a suggestion from Professor Reighard, devised a modification of the above method which has proved a valuable supplement to it.

As soon as Amœba appears in the Ceratophyllum cultures, skim off the brown scum with a spoon, and put it in small bacteria dishes about four inches in diameter and one and one half inches deep, with enough water to fill the dish about one inch deep. Add a little of the decaying vegetable material from near the surface of the Ceratophyllum cultures. This should be done several days before the Amœbæ are desired for use. Cover the dishes and keep them in a *warm* place, but not in direct sunlight. In a few days Amœbæ will be found in abundance in some of these cultures, in the scum at the surface or in the ooze and on the decaying plant material at the bottom of the dish. Some of the cultures prepared in this way by the writer gave results far better than were obtained from the ordinary Ceratophyllum cultures; the specimens were remarkably large, active, and numerous. The Amœbæ pass through stages of development and sometimes practically all of them in a given culture encyst at the same time. They may reappear later in an active stage. Later on, among other organisms may appear in these cultures Arcella, Chilomonas, Paramœcium, Vorticella, and occasionally some colonial Protozoa.

2. *Arcella and Difflugia* will often be found in ordinary Amœba cultures, or in cultures made with dead water-lily leaves. In rather fresh cultures, these Protozoa may be found creeping along the surface of the vegetation. Take up a mass of the Ceratophyllum between the fingers and bruise it briskly against a watch glass; add a *little* water to the residue that clings to the glass. It will usually contain Arcella and Difflugia.

3. *Chilomonas* may appear at almost any time in Amœba cultures; but it is best to put up cultures especially for Chilomonas and Infusoria. Use Ceratophyllum and partly decayed water-lily leaves, and pack in bacteria dishes as for Amœba, but use less plant material and more water. Cultures prepared in this way may mature in a very few days. Mount a drop of the water containing Chilomonas, together with a little of the slime or decaying vegetable material by means of which the specimens may be entangled.

4. *Euglena* will appear in some of the cultures put up for Amœba, usually after the disappearance of Amœba, four or five weeks from the date of starting the cultures, but occasionally it appears before Amœba. It makes a solid deep-green (not blue-green) mass at the edge of the dish, especially on the side towards the light. A thick, pale brown, felt-like and granular scum on the surface of the culture seems to be favorable to the presence of Euglena. Such a scum contains delicate fungi, diatoms, desmids, Arcella, etc.

Euglena usually appears in only a few dishes, but in abundance, and lasts several days. Euglena is often associated with Phacus, which somewhat resembles it, but the latter organism is skate-shaped, and does not exhibit euglenoid movements.

Keeping the cultures away from a strong light may prevent the formation of an excess of chlorophyll in Euglena.

5. *Paramœcium.*— Prepare cultures in the same manner as for Chilomonas. Decaying water-lily leaves are especially good. The cultures require but a few days to mature, and last a long time. Mount a drop of water containing Paramœcia, together with a little of the decaying vegetable material about which they may collect.

6. *Vorticella* is often found on decaying duckweed, Riccia, and Ceratophyllum. Place a large handful of the material in a bacteria dish nearly filled with water. Vorticella often appears in cultures prepared for other purposes.

7. *Carchesium* often occurs on water snails and crustaceans amongst duckweed and Riccia. Collect in the same manner as Vorticella.

8. *Volvox* has been found to occur most abundantly amongst duckweed and Riccia, in pools or small ponds that do not dry up. Collect this material by dipping it up together with a little of the water and place a small handful of the material, with some of the water, in each of many bacteria dishes and add enough tap water so that the dishes are nearly full. Place the dishes so that one side is strongly exposed to light. After a few hours, specimens of Volvox may be seen with the naked eye, or with a hand lens, at the water's edge on the lighted side of the dish. Remove them with a pipette to a small covered dish filled with clean water. Volvox kept in clear water and strong light seeks the bottom of the dish, and can readily be found and removed with a pipette when wanted. If left in the original dish, there may be present small crustaceans which will eat them; yet Volvox may sometimes be collected from these dishes for several days. Usually the material yields best after a day or two, and in three or four days becomes worthless. If obtained free from the animals that prey upon it, Volvox may be kept for several days or even longer.

Volvox is not always present in localities that seem favorable for it, and ponds that contain it in abundance are somewhat rare. In some situations it may be collected to advantage by sweeping a bolting-cloth net over water plants, or, better, using a "Birge net," which has a coarse net over the top for keeping out large forms and trash.

Volvox may be preserved in 4% formalin. Formalin specimens show flagella better than living ones. On account of the uncertainty of obtaining Volvox late in the fall, a supply of preserved specimens should be laid in early in the season.

In studying Volvox, mount the specimens in hollow slides or on ordinary slides with cover-glasses supported by fine glass rods or by bits of broken cover-glass.

9. *Pandorina* sometimes forms a green scum on the surface of the water amongst water lilies, or on the lily leaves.

10. *Hydra* occurs most abundantly on the following plants: duckweed, Riccia, Ceratophyllum, Elodea, watercresses. Two methods of collecting may be used. (a). Method best adapted to duckweed and Riccia: bring in the material with enough water to keep it moist. Fill a large number of bacteria dishes nearly full of clear water. Place in each dish a *small quantity* of the plant material — scarcely enough to cover the surface of the water on which it floats. Place each dish so that one side will be exposed to the light. A day or two later hydras will be found in the extended condition, clinging to the side of the dish, especially the lighted side, to rhizoids of plants, or to dead plants and sediment at the bottom. They may be removed from the sides of the dish with a pipette, or if clinging to plants, the latter may be removed with forceps. Place the hydras in a covered dish of clean water with a small amount of actively growing plant material. Here they will keep indefinitely and may readily be found when wanted. It is not safe to leave them in the original dishes, as there may be present crustaceans which will eat them, or an excess of vegetable matter may cause the water to become foul, which is unfavorable for Hydra. However, the dishes should be inspected daily, for sometimes the material will yield Hydra indefinitely.

(b). When Hydra is to be obtained from large plants like Ceratophyllum and Elodea, the best method is as follows: place a considerable quantity of the plant material in each of several large bacteria dishes and cover it with water. In a short time the water becomes foul through the decomposition of vegetable matter; the hydras then loosen their hold upon the vegetation and may be found floating at the surface of the water. They must be picked off at once with a pipette and removed to clean water.

Hydra will reproduce readily by budding if kept in a fairly warm room; but the optimum temperature is not very high. It is said that if Hydra is kept in the dark at a slightly lower temperature than usual for several days, this will favor the formation of spermaries and ovaries. Rarely, a mature fertilized egg in the winter condition may be found.

11. *Planarians* are found on the under side of stones in running water. They are usually abundant in a locality if found there at all. They may be removed from the stones by means of a thin wooden toothpick, and placed in a bottle of water for transportation.

12. *Earthworms.*—The *collection* of earthworms should be attended to as early in the fall as possible, as dry or cold weather may make it impossible to get them later. It is well to lay in a supply of preserved worms during the spring or summer. Specimens to be kept alive should perhaps not be collected until after the first of October, but it will be unsafe to wait much longer. Each student will need two or three preserved, and one or two living specimens.

The form used in the Zoölogical Laboratory of the University of Michigan is *Lumbricus herculeus* Savigny. The specimens range in length from about 6 to 11 inches, with an average of about 8 inches. They come out of their burrows on warm, rainy nights, usually lying extended with the posterior end of the body still in the entrance of the burrow. They are most abundant in old, rich gardens and lawns. Go out with a lantern and a pail after it has become quite dark; the harder it rains the better. By stepping lightly and not allowing the light to shine upon them too long, one may seize the worms with the hand. To catch them requires quickness and dexterity; but they must be pulled gently from the burrow.

When brought into the laboratory the worms that are to be preserved for dissection should be placed immediately in covered bacteria dishes (9 inches in diameter by 3 inches deep) between sheets of moist filter paper; the covers should be adjusted so as to admit a little air. Not more than twelve specimens should be placed in a dish, as they will soon die if large numbers of them are left together. The dishes should be kept in a cool place, avoiding direct sunlight. In the morning the paper should be changed, and injured or dead specimens removed; the operation should be repeated as often as is necessary. After a day or two the worms will have eaten enough moist filter paper to clean the alimentary canal of earth, and are ready for preservation.

Worms to be kept alive for a considerable length of time[1] may be placed, immediately after collection, between folds of moist muslin in bacteria dishes, with not more than a dozen specimens in a dish. Change or wash the cloth occasionally — at least every two weeks. The worms may be fed on leaves, etc., if desired. With proper care, they will keep in this way for months, and are always clean and ready for use at a moment's notice. The writer has found that the worms will keep quite as well if filter paper is used instead of muslin.

Preparation of Earthworms for Dissection.— Fresh specimens are too soft for convenient handling of the tissues during dissection; it is also inconvenient to keep a sufficient number of them alive for general dissection purposes; hence preservation is resorted to. Two methods are available: —

(*a*) *Chromic Acid Method.*

1. *Killing.*— Place in 4 % alcohol and gradually increase the strength to 8% in the course of the next two hours by adding stronger alcohol a few drops at a time. Wash in water to free from mucus and again immerse in 8 % alcohol until the worms are thoroughly stupefied and no longer contract when pinched with forceps.

2. *Fixing.*— Inject worms with 1 % chromic acid and immerse in the acid for about 4 hours.

The injection is best performed by means of a water pressure apparatus (see Fig. 1). This gives constant, uniform pressure. A head of about four feet of water should be used. A glass cannula may be made by drawing out the end of a piece of $\frac{1}{4}$ inch glass tubing to a very fine bore, and breaking it so as to leave a sharp point with a raw edge. The exact size must be determined by experiment. The stream may be allowed to flow continuously from the cannula. A pipette with point drawn out fine may be used in case this apparatus is not available, but it is not very satisfactory.

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[1] H. S. Jennings. "Keeping Earthworms Alive in Winter." *Journ. of Applied Micr.*, vol. 6, no. 7, pp. 2412–2413.

Fill a large shallow glass dish about ½ inch deep with 1 % chromic acid. (Cover the hands and wrists thickly with vaseline while working with chromic acid.) Take each worm separately and lay it in an extended condition in the chromic acid, keeping it straight and seeing that it is not twisted. Inject immediately, about ½ inch behind the clitellum, and again near the posterior end of the body if necessary. *Inject into the body cavity; be careful not to pierce the alimentary canal.* The worm should swell out slowly along its entire length and become very turgid. Too rapid injection may tear the tissues.

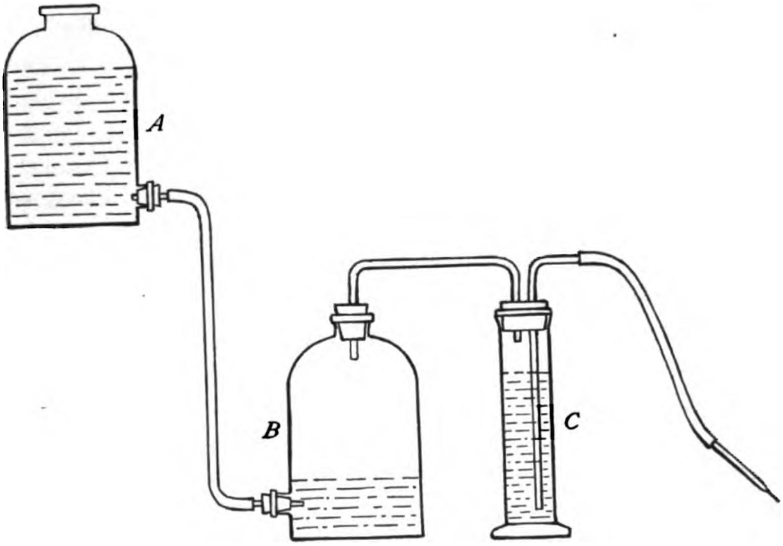

FIG. 1.— Constant pressure injection apparatus. *a* should be filled with water, part of which flows into *b*; *c* contains injection fluid.

The worms should be kept straight while in chromic acid. If left in the acid less than four hours they will eventually become too soft; if left in the acid much more than four hours they will be too brittle.

Wash thoroughly in running water until the yellow color is gone — about 12 hours. Be sure that the water reaches every part of the surface of each worm. If free acid remains on the worms it will make them brittle. The worms are a light drab, not yellowish brown, when well washed.

3. *Preserving.*— Place the worms in 50% alcohol and leave for a day or two; the alcohol will become discolored by chromic acid. Change to 70% and leave a day or two longer. Then change to the same grade (70%) for final preservation.

The alcohol will complete the process of removing free acid and will further harden the tissues. Stronger grades of alcohol than 70% are not advisable for preservation, as they may cause the more delicate tissues to become brittle.

(*b*) *Alcohol Method.*

Kill as directed under chromic acid method. Place the worms in 50% alcohol, keeping bodies as straight as possible. Leave 4 or 5 hours, then place in 70% alcohol and leave over night. Next morning place in 96% alcohol to harden them, and leave all day. Change to 70% alcohol for final preservation.

Choice of Methods.— Specimens hardened in chromic acid are most satisfactory for general dissection purposes. The tissues are firm and leathery, and are not affected by water during dissection, hence they may be dissected under water instead of alcohol. This is a decided advantage. The largest and best marked specimens should be put up in this way.

The alcohol method of fixing is not so satisfactory for most purposes; the body wall and tissues generally are too soft to be conveniently handled unless the dissection is done under alcohol. But the method has the advantage of simplicity, and the smaller and more poorly marked specimens may be put up in this way for use in case other material runs short. Alcoholic specimens do very well for the study of the nervous system.

Fresh specimens are not only necessary for the study of the living worm, and for demonstrations of cilia, blood, cœlomic fluid, spermatozoa, etc., but are best for the study of the circulatory system and the nephridia.

For studying the circulatory system the living specimen may be stretched out in a dissecting tray and fastened with a pin through each end of the body; if desired it may first be stupefied with alcohol. The dissection should be carried on under normal salt solution (0.75 % NaCl). In the absence of living specimens, chromic-acid specimens do fairly well for the circulatory system.

For sectioning with a microtome it is necessary to have specimens with intestines entirely free from grit. Worms that have been kept in clean, moist muslin for a considerable length of time will usually be found to have the alimentary canal free from all undesirable substances. The muslin should be changed every day, and the worms must not be fed. A quicker method[1] is to flush the alimentary canal by means of an injection apparatus. Use a cannula with a rather large opening; the proper pressure must be determined by experiment. Stupefy the worms with dilute alcohol (3 to 6 %). Inject the alimentary canal from the posterior end with 0.75 % salt solution; roll the body of the worm back of the clitellum between the fingers and strip out the contents of the intestine. Then inject from the anterior end; this time the stream will go entirely through. Avoid too much pressure, as it will injure the tissues. Roll and knead the body while the stream is being started through. The fluid should be forced through the worm until it comes from the posterior end in a perfectly clear stream. The worm is then ready for fixing.

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ANN ARBOR, MICH.

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[1] Raymond Pearl. *Journ. of Applied Micr.*, vol. 3, no. 1, p. 680.

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PRELIMINARY REPORT ON THE EMBRYOLOGY OF CRYPTOBRANCHUS ALLEGHENIENSIS

BERTRAM G. SMITH

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PRELIMINARY REPORT ON THE EMBRYOLOGY OF CRYPTOBRANCHUS ALLEGHENIENSIS.[1]

BERTRAM G. SMITH.

Although the eggs of the hellbender, *Cryptobranchus* (*Menopoma*) *allegheniensis*, have been eagerly searched for by embryologists, a few unfertilized eggs only have hitherto been obtained. Aside from a brief description of an embryo in an advanced stage by McGregor ('96), there is no record of any observations on the development. The manner in which the eggs are fertilized has not been described.

This gap in our knowledge of comparative embryology is serious, particularly as very little is known about the development of the two other members of the family[2] Amphiumidæ: the giant salamander of Japan, *Cryptobranchus japonicus* (*Megalobatrachus maximus* Schlegel), and the American *Amphiuma means*.

During the early part of September, 1905, in northwestern Pennsylvania, I was so fortunate as to obtain fertilized eggs of *Cryptobranchus allegheniensis* in abundance, both from specimens in captivity and from the natural habitat of the animal.

I. The Sexual Elements.

A. *The Egg.*—The egg proper is perfectly spherical when fresh, about the size of a large pea, and bright yellow in color—a rather deep yellow at the lower pole, grading to a pale yellow at the upper. There is no black pigment such as is found in the eggs of most amphibians. A very thin transparent membrane, the vitelline membrane, quite inconspicuous in fresh material, closely invests the egg. Each egg, with its vitelline membrane, floats in a clear fluid within a hollow sphere or capsule about the size of a large grape, formed by the thick gelatinous outer envelope, the secretion of the oviduct. This outer egg envelope is produced at opposite poles of the capsule to form a slender cord connecting the eggs in a string (Fig. 1). The envelope is

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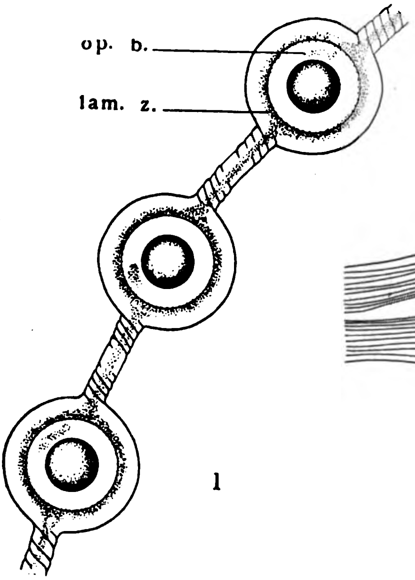
[1] Contributions from the Zoölogical Laboratory of the University of Michigan, No. 105.

[2] Gadow's classification is followed ('01).



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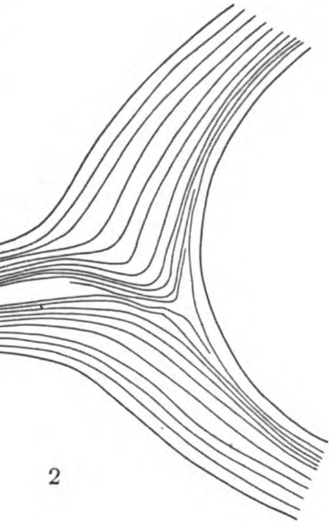

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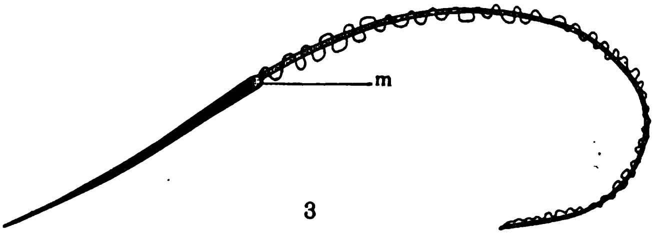

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FIG. 1. Eggs and egg envelopes of *Cryptobranchus*, natural size. *op.b.*, opaque body ; *lam.z.*, lamellar zone of envelope.

FIG. 2. Optical section (longitudinal) through lamellar zone of envelope in region of junction of egg capsule with connecting cord. Magnified about thirteen diameters.

FIG. 3. Spermatozoön, magnified about five hundred diameters. *m.*, middle piece.

perfectly transparent when fresh, except that wherever viewed tangentially its inner layers have a milky appearance, represented by the shaded zone in Fig. 1, and due to a fine lamellar structure sketched in optical section, with a magnification of about thirteen diameters, in Fig. 2. The misty appearance is caused by the diffusion of light passing through these concentric layers tangential to their inner surfaces. The core or axis of the connecting cord also has the same milky appearance, due to a continuation of the lamellar structure.

The outer surface of the capsule and cord is, to ordinary observation, perfectly smooth and regularly rounded when the material is in fresh condition. But a careful examination, especially with the aid of a lens, reveals a delicate longitudinal striation due to fine parallel grooves or scratches separated by nearly equal intervals. This structure is too minute to be represented in Fig. 1. In some portions of the string the striæ have a slightly spiral direction about the axis of the cord and capsule. The striæ appear as they would if made by the scratching of the surface of the envelope against the teeth of a fine comb; perhaps they are caused by friction of the envelope against papillæ of some sort in the lower portion of the oviduct or in the cloaca, while the gelatinous material is yet soft.

After the eggs have been in water for several days, or after preservation in formalin or alcohol, the outer layer of the egg envelope becomes cast into conspicuous wavy folds or wrinkles of a different nature from the striæ noted above. These folds appear first at the ends of the connecting cord adjacent to the egg capsule and have here a strongly marked spiral arrangement, suggestive of the chalazæ of the hen's egg (see Fig. 1); later the folding may extend over the capsule and the middle of the cord, but seldom with so regular a spiral arrangement in these regions. Sometimes the spiral is continuous from one capsule to another. As a rule the spiral is constant in the direction in which it extends about the axis in all portions of the cord and capsule.

The inner layer of the lamellar core of the cord in some cases exhibits a marked twisted or spiral arrangement like that of the inner portion of the cord connecting the eggs of *Ichthyophis* as described by the Sarasins ('87–'93).

The following measurements were taken :

(1) Diameter of egg proper,	7 mm.
(2) " " " with envelopes,	18 "
(3) " " connecting cord,	5 "
(4) Distance from one egg to another, measured from center to center,	3 cm.

These figures represent the average of several measurements, the range of variation of the first dimension being small, of the others considerable, in eggs from the same parent. The egg proper ordinarily sinks to the bottom of the fluid which surrounds it, so that when viewed from above it is magnified by the lens-shaped capsule so as to appear considerably larger than is indicated by direct measurement.

Occasional strings of egg capsules are found without any eggs enclosed. These empty egg envelopes have the same structure as the others, and usually occur as a portion of a string of capsules the remainder of which contains eggs. A few of the empty egg capsules are double, formed by the union of two capsules without a connecting cord; in such cases the cavities of the two capsules are separated only by a very thin gelatinous septum.

In fertilized eggs in an early stage of development, preserved in formalin without removal from their envelopes, I found floating in the liquid between the egg and its envelope a fairly large irregular and slightly opaque mass, in appearance like a faint white cloud (see Fig. 1). This mass had not been noticed in the capsule of the living egg, probably because it was then transparent. Under the microscope it was found to consist of a clear gelatinous matrix in which were embedded numerous large round cells each with a very large nucleus and often with what appeared to be a large vacuole. By the use of a dissecting lens the individual cells could be seen while still within the envelope. In some egg capsules they occur diffused throughout the liquid of the cavity, not aggregated in a mass to form an opaque body. Numerous spermatozoa are present in the opaque body, but they also occur floating in the surrounding liquid and embedded in the egg capsule. Whether the opaque body is present in unfertilized eggs I have not at present the material to determine. The unfertilized eggs of *Cryptobranchus allegheniensis* have been briefly described by Reese ('04) but without mention of any such feature as the opaque body described above. Both opaque body and spermatozoa regularly occur in egg capsules that do not contain eggs. The origin and function of the cells of the opaque body are at present problematical.

The eggs of *Cryptobranchus alleghenienis* bear a rather close resemblance to those of *C. japonicus* as described by Sasaki ('87), Kerbert ('04), Ishikawa ('04) and de Bussy ('05). Other amphibians whose eggs are fastened together like a string of beads are *Amphiuma* (Hay, '88 and '90), *Desmognathus*, *Ichthyophis*, *Hypogeophis* (Brauer, '97), and *Alytes*. The eggs of *Bufo* are arranged in strings but without the marked constriction of the envelope between the eggs characteristic of the other forms mentioned.

B. *The Sperm.* — The spermatozoön (Fig. 3) is of considerable size, about 225 μ long, but with an unusually small middle-piece. The head of the spermatozoön, excepting the tip or acrosome, stains deeply with Conklin's modification of Delafield's hæmotoxylin. The head is very long and slender, tapering gradually to the extremely fine-pointed acrosome, enabling the spermatozoön to pierce easily through the vitelline membrane. The tail-piece is provided with an undulating membrane, bordered with a convoluted filament.

The spermatozoön of *Cryptobranchus alleghenienis* resembles that of *C. japonicus* as figured by Ishikawa ('04). It is also quite similar to the spermatozoön of *Amphiuma* (McGregor, '99), but the latter has a larger middle-piece and a slightly barbed acrosome.

II. Breeding Habits.

A. *Method of Fertilization.* — A newly-captured female was isolated in an aquarium not previously occupied by any other specimen. A large number of eggs were laid, which contained spermatozoa within the egg capsule; moreover the subsequent development of these eggs proved them to have been fertilized. Hence there can be no doubt that in *Cryptobranchus alleghenienis*, as in all other Urodeles so far as known, fertilization, unlike that of the *Anura*, is internal. The mode of transference of the male element to the cloaca of the female has not been ascertained.

Nothing conclusive has been established concerning the method of fertilization of the nearest relative of *Cryptobranchus alleghenienis*, the giant salamander of Japan. In the case of *Amphiuma*, Davison ('94 and '95) believed a transference of spermatozoa from male to female to take place by means of an apposition of the lips of the two cloacæ, basing this belief on what appears to me insufficient evidence; such a process is moreover not in harmony with known methods of fertilization among the Urodeles, in which fertilization is effected by means of spermatophores and without direct cloacal contact (Jordan, '91 and '93). Internal fertilization is said to occur in the Gymnophiona, *Ichthyophis* and *Hypogeophis*, as well as in the Urodeles.

B. *Breeding Season.* — Fertilized eggs were laid by a specimen in captivity on September 6, and another spawning by the same female took place during the night of September 7–8. Eggs in the first cleavage stage were found in the natural habitat of the animal on September 7, and another spawning of eggs in an advanced stage of segmentation was found in the same habitat on September 8.

Townsend ('82) records the laying of some eggs by a specimen in captivity during the month of August. McGregor ('99) states that the eggs are deposited in August and September.

No direct observations as to the time when the females are fertilized were obtained; but male specimens killed during the early part of September were found to have the vasa deferentia distended by a large quantity of seminal fluid containing an abundance of ripe spermatozoa. According to McGregor ('99) "the sexual union must occur very near or at the time of egg-laying, for the female is devoid of spermathecæ, and the spermatozoa do not ripen until late in August."

Eggs of *C. japonicus* were found by Sasaki ('87) in August. Kerbert ('04) records the spawning of a specimen in captivity during the night of September 18, 1902, and again on September 19, 1903. DeBussy ('05) reports that eggs were laid by Kerbert's specimen during September 14, 1904, and several days following. According to Ishikawa ('04) the eggs are laid principally during the latter half of August, but also in September. Hence the breeding season coincides with that of *C. alleghaniensis*.

Eggs of *Amphiuma* in an advanced stage of development were found by Hay ('88 and '90) on September 1. Davison ('94 and '95) states that the eggs are deposited in August or September, but without giving the data upon which this statement is based.

He found a viscid substance containing spermatozoa exuding from the vent of a male specimen in May (April, according to his earlier paper), and concluded that this month is the natural season for fertilization.

The occurrence of the breeding season of *Cryptobranchus* in the fall is in marked contrast to the habits of nearly all other Urodeles, since they lay their eggs in the spring. While the significance of this unusual breeding season is not readily apparent, it is, in the case of *C. allegheniensis*, in at least one respect adaptive. The animal is an inhabitant of streams that during spring and early summer are subject to frequent and destructive freshets, which would probably be disastrous to the development of eggs like those of *Cryptobranchus*. During late summer and fall the streams are shallow and the water comparatively quiet; floods are of rare occurrence. These factors do not affect the other amphibians of the region in the same way, since they are inhabitants of ponds not seriously disturbed by floods, and on account of the more abundant rainfall better adapted for breeding-grounds in the spring than later in the year. There is every reason to believe that these climatic conditions have been of long duration. Probably the same conditions prevail with regard to *C. japonicus*, which is an inhabitant of mountain streams similar to those in which *C. allegheniensis* occurs. In this connection Professor Jacob Reighard informs me that the increase of spring freshets in Michigan during recent years, aided no doubt by other effects of lumbering operations, has nearly caused the extinction of the grayling, a fish that breeds in the spring, and was formerly abundant; the trout, which breeds in the fall, now thrives in the same streams. These facts indicate the selective value of the factors mentioned, and support the view taken with regard to *Cryptobranchus*.

C. *Breeding Habitat.* — Eggs of *Cryptobranchus* were found in shallow water in what had once been the main channel of a large stream, but through which now only a portion of the water, separated from the main channel by an island, flows. This old river channel is extremely rocky, with a considerable incline, so that the shallow water alternately forms pools and rapids. Judging from the number of specimens seen, the locality is a favorite haunt of *Cryptobranchus*. Adult hellbenders were seen in other portions of the stream in deeper water, but in no other situations were eggs found.

The locality described above bears a strong resemblance to the habitat of *C. japonicus* as illustrated by Ishikawa ('04).

D. *Habits of Oviposition.* — Two separate spawnings of eggs were found in the natural habitat of the animal, besides eggs laid on two different occasions by a specimen in captivity. On account of differences in the method of disposal of the eggs, these spawnings are best described separately.

The eggs found on September 7 were lying in gently flowing water about 2–4 inches deep, on a gravelly and stony bottom, within a space about 6 feet in diameter nearly enclosed by some large rocks which projected a foot or more out of the water. The eggs were arranged in long festoon-like strings, scattered over an area of about 2 x 5 feet. In a few places the eggs were grouped in masses, but these masses might readily be resolved into strings. All the eggs present were included in a very few strings; one string contained 27 eggs. Evidently the eggs had not been disturbed since being laid. One hundred and ten eggs were counted in full view; but some other eggs had sunk down into crevices between and beneath stones; these brought the entire number up to 135. The eggs lay for the most part in direct sunshine, and the shallow running water furnished them with abundant aëration. No adult hellbenders were seen in the immediate vicinity.

The eggs were conspicuous because of their size and number, and were really beautiful objects, on account of the regularity of their form, the festoon-like manner of their arrangement on the pebbly bottom, the bright yellow of the yolk, and the perfect transparency of their gelatinous envelopes except where bordered with a delicate misty gray.

On September 8 another spawning of eggs, perhaps 50 in number, was found not far from the spot where eggs were discovered on September 7. The eggs were in water 3–5 inches deep, on the down-stream side of a rock, and most of them in a shallow cavity about 16 inches wide and extending back about 8 inches under the rock. From the appearance of the surrounding and underlying gravel this hollow looked as if it had been dug out by some animal. The eggs had been disturbed and scattered considerably; the strings were short, as if they had been much broken up, and many separated eggs were found. The eggs were thickly covered with silt. Both the envelopes and the contained eggs were, as a rule, slightly larger than those previously found. No adults were seen in the vicinity.

The eggs obtained on September 6 from a specimen in captivity were found rolled and tangled together in such an intricate manner that they seemed to occur in clusters or masses rather than in strings. The number was estimated at about 80, nearly all in one oblong mass. The solitary female did not appear to care for the eggs in any way.

On September 8 about 300 more eggs were laid by the same female specimen in captivity. The strings of eggs were aggregated in one large mass, but they were not so much tangled as in the case of those laid previously. There were present at the time in the same aquarium three male specimens, but so far as was observed, none of the adults ~~paid any attention~~ to the eggs. When all four specimens were killed a few days later for the purpose of determining the sex by dissection, the stomachs of the males were found distended with undigested eggs.

Upon examination, the female, which during captivity had laid nearly 400 eggs, was found to contain, at a rough estimate, seven or eight hundred more, in a state of development which indicated that had she been allowed to live they would all have been laid during the same season. Evidently the eggs are, in some cases at least, matured and laid in batches of a few hundred at a time.

According to Gadow ('01), *Amblystoma* alone among the Urodeles lays as many as 1,000 eggs in a single season. The facts stated above make it probable that *Cryptobranchus* rivals *Amblystoma* in the number of eggs laid. Kerbert's specimen of *C. japonicus* is reported to have laid 500 eggs in the fall of 1902, and about 900 in 1904. The eggs of *Amphiuma* found by Hay numbered about 150.

The absence of any evidence of brooding habits of either the male or the female *Cryptobranchus* is rather unexpected in view of the possession of brooding habits by closely related forms.

Concerning the nesting and brooding habits of *C. japonicus* Ishikawa says: "Das tier legt seine Eier in tiefe horizontal verlaufende Löcher, in denen das Wasser sehr ruhig ist. Manchmal ist solch ein Loch 10 oder mehr Fuss tief und kaum für das Licht zugänglich. Die Brutstellen für die Eier sind aber nicht immer so tief. Oft fand ich Eier in einem Loch nicht tiefer als 3 oder 4 Fuss. Oeffnet man ein solches Loch, so findet man eine abgerundete Stelle, deren Boden ganz rein gehalten ist. . . . Fast in jedem Loch, wo man von Ende August bis zu Anfang October ein weibliches Tier gefunden hat, findet man einen Eiklumpen. Dieser Umstand lässt schon vermuthen, dass das Tier eine Brutpflege hat wie *Ichthyophis* oder wie so viele andere Amphibien." Kerbert, however, asserts that it is the male that guards the eggs, and states that the sex of his specimens was carefully determined. The only external distinction between the sexes is that during the breeding season the lips of the cloaca of the male are greatly swollen. Ishikawa gives an illustration of an adult specimen of *C. japonicus* lying in a coil about a mass of eggs; Kerbert states of his specimen that the male, after driving away the female and also the small fishes present in the aquarium, crept between the folds of the mass of eggs, or sometimes simply lay down beside them, but in either case he kept the entire mass in motion by a pendulum-like movement of the entire body.

The eggs of *Amphiuma* found by Hay ('88 and '90) in an Arkansas swamp were in a comparatively dry situation, in a small excavation under a log several rods from the nearest water. The female was found coiled about the mass of eggs, protecting them and keeping them moist.

Other Amphibia for which brooding habits have been established are the Urodele, *Desmognathus;* the Gymnophiona, *Ichthyophis* and *Hypogeophis; Alytes* and several other Anura (Wiedersheim, '00). In the cases of *Desmognathus*, *Ichthyophis* and *Hypogeophis*, the female is said to care for the eggs; in the case of *Alytes*, the male.

E. *Adaptation of the Egg to its Environment.*— The egg proper is so soft and fragile that it can sustain only the most careful manipulation without injury; on account of its lack of firmness it soon becomes oblate from the effects of gravity. In view of the fact that the eggs are laid in running water, the protection of such an egg from shocks and jars due to impacts against the rocks is an exceedingly important matter, and we find it accomplished by an admirable adaptation of the egg envelope. The tough but elastic egg capsule is inflated by osmosis with a liquid in which the egg freely floats. The turgid condition of the envelope increases its efficiency in protecting the egg. While ordinarily the egg sinks to the bottom of the surrounding liquid, and rests lightly upon the membrane beneath, there is so little friction between the egg and its envelopes that the latter may be rotated without turning the egg. A similar adaptation exists in the eggs of other amphibians, and in the eggs of teleosts (Reighard, '93); but it is particularly well shown in the egg of *Cryptobranchus*. The connecting cord is strong enough to support the weight of half a dozen eggs suspended out of water; it stretches greatly during the operation, but contracts to its usual length when the eggs are returned to the water. This elasticity of the connecting cord serves to deaden any shock to the eggs due to the tugging of the current or to the chain catching on to rocks while floating down stream.

The egg envelopes further serve to protect the eggs from little fishes that would otherwise devour them.

If eggs are left for several days undisturbed in the still water of a basin, some of them adhere to the envelopes beneath, interfering with the development. Probably under natural conditions the gentle agitation of the current tends to prevent this, performing for these eggs the same service afforded to the eggs of the hen by the mother when she turns them.

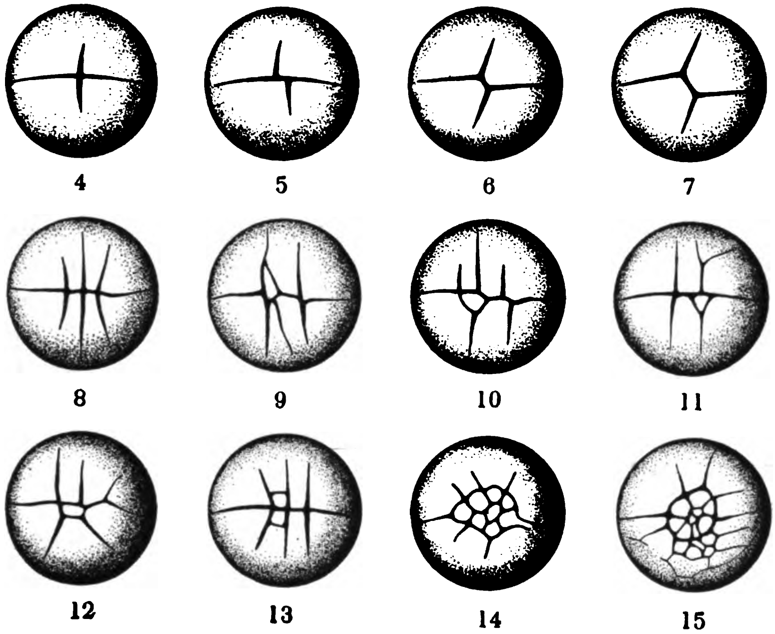
III. SEGMENTATION.

The perfect transparency of the gelatinous envelopes of these eggs makes them very favorable material for watching the development of the living egg, since the process may be observed without removing the egg from the capsule. The large size of the eggs and the distinctness of the cleavage lines on their upper hemispheres also make them convenient objects for study. By using a mirror both poles of the egg may be watched at the same time, but observations on the lower hemisphere by this method are rather difficult, on account of the faintness of the cleavage lines in this region. Material was preserved at intervals and camera drawings afterwards made of the principal stages; but for the earliest stages the freehand sketches made from living material are sufficiently accurate, and have been used in preference to camera drawings from preserved material.

Some of the eggs found on September 7 were left to develop *in situ* as a check upon those studied under artificial conditions; but so far as cleavage is concerned no difference could be detected between these eggs and those kept in a dish of shallow water.

The abundance of the material at hand made it possible to secure a series of drawings of the segmentation stages which should be fairly representative. The drawings include some of the most regular and geometrically perfect figures that could be found, and probably these may be regarded as typical; but in *Cryptobranchus* as in other amphibians the amount of variation is great. Since departures from the type are not necessarily the result of pathological conditions, they should not be dismissed as abnormalities; for they may be the expression of opposing factors in the development of the egg. Such factors are the proportion of yolk to protoplasm, the qualitative composition of the yolk determining the extent to which it responds to the sorting influence of gravity, and hereditary factors in the protoplasm; variations in these factors affect the manner of cleavage, and according as one tendency or another prevails we may find the typical form or variations from it in definite directions. Hence the figures include representations of eggs in the same stage showing differences in the method of cleavage. There are of course individual differences in the rate as well as in the manner of cleavage.

A. *The First Cleavage Furrow.* — In eggs laid September 6 at about 6:30 P. M., there was so much diversity in the time of appearance of the first cleavage furrow that it is difficult to assign limits. Probably more cases of first cleavage were noticed at about 10 A. M. of the next day — 15 hours after the eggs were laid — than at any other time. In eggs laid during the night of September 7–8 the first cleavage furrows appeared in several eggs at about 5 P. M. of the next day, and cases were numerous at 6:30 P. M. The eggs found on September 7 at 10 A. M. were in the first cleavage stage; observations on the rate of deposition of silt on their envelopes indicated that they had been laid during the preceding night or late in the afternoon of the day before.



FIGS. 4–15. Early segmentation stages of eggs of *Cryptobranchus*. Freehand sketches from living material magnified about 3 diameters.

FIG. 4. Egg found September 7 at 10 A. M., sketched at 1 P. M. The first cleavage line extends over exactly half a circumference.

FIGS. 5–7 show variations in the second cleavage. Fig. 5, egg laid September 6 at about 6:30 P. M., sketched September 7 at 3 P. M. Figs. 6 and 7, eggs found September 7 at 10 A. M., sketched at 1:30 P. M.

FIG. 8. Egg laid during night of September 7–8, sketched September 9 at 5:30 A. M.

FIGS. 9–11. Variations in third cleavage. Eggs laid during night of September 7–8, sketched September 9; Fig. 9 at 3:50 A. M., Fig. 10 at 2:30 A. M., Fig. 11 at 5:30 A. M.

FIG. 12. Egg laid during night of September 7–8, sketched September 9 at 5:45 A. M.

FIGS. 13 and 14. Fourth cleavage stages. Fig. 13, a later stage of egg represented in Fig. 8, sketched September 9 at 8 A. M.; Fig. 14, a later stage of egg represented in Fig. 9, sketched September 9 at 8 A. M.

FIG. 15. Egg found September 7 at 10 A. M., sketched September 8 at 8:45 A. M. Four nearly equidistant cleavage lines reach almost to the center of the lower pole.

It is not known whether the eggs of a given spawning are all fertilized at the same time, hence some of the diversity in the time of appearance of the first cleavage furrow may be due to a difference in the time of fertilization.

The first cleavage furrow begins as a pit, which gradually elongates, rapidly at first then more slowly. The point of origin of the first cleavage furrow remains always uppermost, even though the envelope is rotated.

B. *The Second Cleavage Furrow.* — This furrow makes its appearance about six hours after the first, which by this time has extended over about half the distance to the equator of the egg. The second cleavage furrow usually cuts the first at right angles, but some variations from this procedure are shown in the figures. (See Figs. 4–7.)

Some time before the appearance of the second groove, the first one appears constricted and narrow in the middle portion, while still broad at the ends. The earliest indication of the second furrow is usually a roughness in the region where the second groove is to intersect the first. The appearance of "Faltenkränzen" — a quivering of the surface with the formation of fine radiating or parallel wrinkles, which extend outward from the cleavage furrow for a moment, tremble, and disappear — a common phenomenon in the cleavage of the amphibian egg, is quite marked at the time of the beginning of the second cleavage furrow. For some time after its appearance the second furrow is much broader, though of course shallower, than the first.

C. *The Third Cleavage Furrow* (see Figs. 8–12). — These furrows mark the establishment of the 8-cell stage about four or five hours after the appearance of the second cleavage groove. Hence the third division is more rapid than the second. At the time when the third furrows are initiated the first furrow has usually reached or passed the equator; the second one is confined to the upper hemisphere.

The third cleavage furrows ordinarily begin as two pits in the first furrow, not far from its point of intersection with the second — the center of the animal pole of the egg — and equidistant from that point. From these pits the third furrows proceed in an approximately vertical direction. The third cleavage furrow seldom originates from the center of the first furrow, and seldom reaches the lower pole, but extending obliquely in the lower hemisphere joins the second furrow at some distance from the pole (see plate, Fig. 16*B*, line *b*; Fig. 17*B*, lines *b*, *g* and *e*; Fig. 18*B*, lines *b*, *i* and *e*, etc.). Hence the third cleavage furrow is *intermediate between a true meridional cleavage and a latitudinal one*, approaching more nearly the meridional type. This will be made clearer by supposing the point of origin of the third cleavage furrow to be shifted along the first further from the animal pole; and its point of junction with the second to be shifted further from the lower pole; the third groove will then become latitudinal. On the other hand, if the two ends were shifted in the reverse directions, the cleavage lines would become truly meridional. Variations from the customary mode of cleavage tend to confirm the view that it is an intermediate or transitional form; while some third cleavage furrows originate at the center of the animal pole, giving a true meridional cleavage, others come in latitudinally. (See Figs. 9–12.)

D. *The Fourth Cleavage Furrow* (Figs. 13–15). — This comes in about 3 hours later — a briefer interval than that preceding the appearance of the third cleavage furrow. In its typical condition it is parallel to the equator, but close to the animal pole, so that the division is very unequal. Irregularities in this cleavage are very numerous.

E. *Later Segmentation Stages* (see plate, Figs. 16–24). — The later divisions occur with increasing rapidity, but with such irregularity that no definite arrangement of cleavage furrows can be made out. Cleavage proceeds rapidly near the animal pole, much more slowly in the equatorial region, and is greatly retarded in the lower hemisphere. At some time during the second day after the egg is laid the first cleavage furrow reaches the lower pole. At this time the upper hemisphere is cut up into a considerable number of cells. The macromeres continue to be very much larger than the micromeres. While so far as can be determined from a surface study of the egg, the cleavage is undoubtedly holoblastic, a strong tendency toward the meroblastic condition is evident.

Two days after the egg is laid, cell division in the upper hemisphere has advanced until the individual cells can no longer be seen with the naked eye. The macromeres are in general still quite large, but in several instances there was noted a greater multiplication of cells about the lower pole than in the equatorial region (see Fig. 24*B*).

F. *Comparison with Other Forms.* — De Bussy ('05) has described the principal segmentation stages of the eggs of *C. japonicus.* His material lacked eggs in the 2-cell and 4-cell condition; but beginning with the 8-cell stage the later cleavage stages are described and illustrated. A comparison with my own sketches of the segmentation of the eggs of *Cryptobranchus allegheniensis,* made before I knew of the existence of de Bussy's paper on *C. japonicus,* shows, as might be expected from the close relationship of the two forms, a marked similarity in the mode of cleavage. Since de Bussy's paper includes a comparison of the eggs of *C. japonicus* with those of other forms, a similar discussion for *C. allegheniensis* at the present time seems unnecessary. It should be added, however, that the segmentation of the eggs of *Desmognathus,* described by Wilder ('04) and Hilton ('04) also bears a considerable resemblance to that of *Cryptobranchus.*

I take pleasure in acknowledging my indebtedness for encouragement and advice to Prof. Jacob Reighard, at whose suggestion I first looked for the eggs of *Cryptobranchus,* and to Dr. O. C. Glaser, under whose direction the work was carried on after I returned to the university.

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EXPLANATION OF PLATE.

All the figures are camera drawings from preserved material. Figs. 16–19 are magnified about three diameters, the remaining figures about four diameters. *A* and *B* indicate upper and lower hemispheres respectively. The small letters serve to identify lines which cross the equator.

FIGS. 16–18. Eggs laid during night of September 7–8, preserved September 9 at 2 P. M.

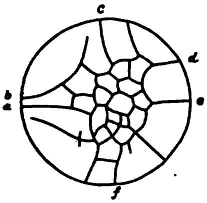
FIGS. 19 AND 20. Eggs found September 7 at 10 A. M., preserved September 8 at 9 A. M.

FIG. 21. Egg laid September 6 at 6:30 P. M., preserved September 8 at 8:30 A. M. Lateral view.

FIG. 22. Egg laid during night of September 7–8, preserved September 11 at 10 A. M.

FIG. 23. Egg found September 7 at 10 A. M., preserved September 10 at 8 A. M.

FIG. 24. Egg laid September 6 at 6:30 P. M., preserved September 8 at 8:30 A. M.



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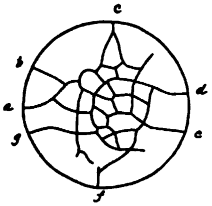

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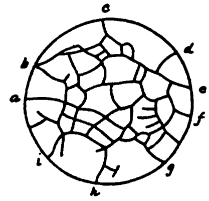

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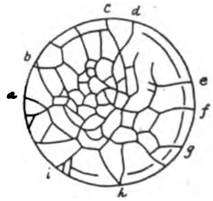

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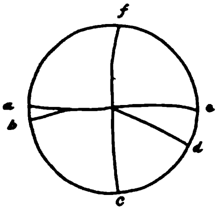

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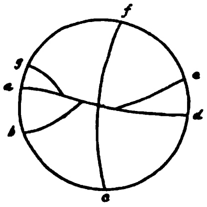

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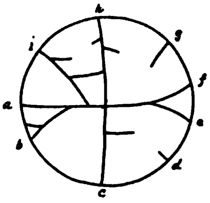

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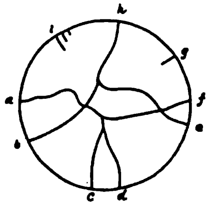

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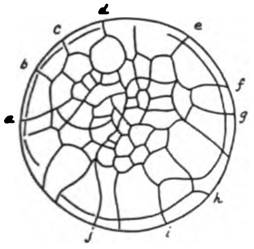

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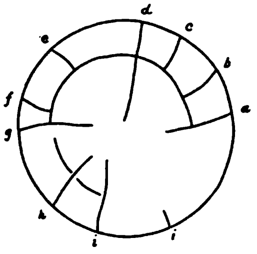

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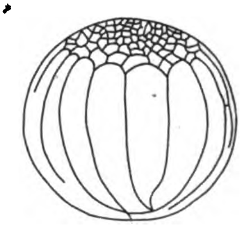

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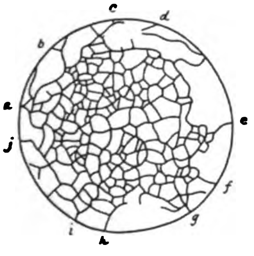

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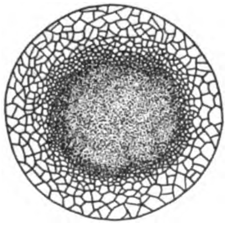

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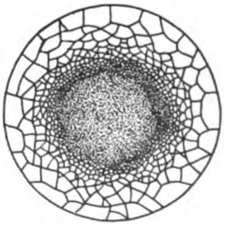

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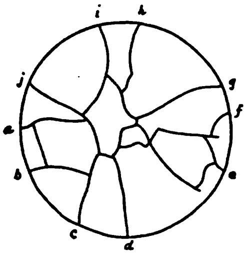

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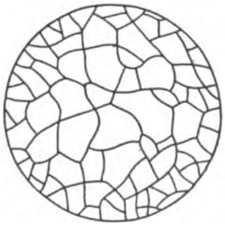

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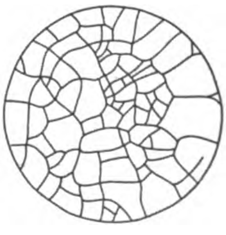

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NOTE ON THE YPSILOID APPARATUS OF CRYPTOBRANCHUS.

A DESCRIPTION of this cartilage in a recent article by Whipple ('The Ypsiloid Apparatus of Urodeles,' *Biol. Bull.*, May, 1906) differs radically from the description by Reese ('The Anatomy of *Cryptobranchus*,' *American Naturalist*, April, 1906). According to Whipple the cartilage has the typical Y-shape common to urodeles, being bifurcated at the anterior end; according to Reese it is rod-shaped. Having an abundance of material at my disposal, I examined this apparatus in a number of specimens. In every case the cartilage is Y-shaped, but with a marked difference in the structure of the anterior and posterior regions: the posterior portion, forming the stem of the Y, consists of a stout rod of cartilage; the expanded V-shaped anterior portion is very thin. In a dry preparation this thin expanded anterior portion would probably shrivel up and might be easily detached and hence overlooked; the remaining portion would then answer the description given by Reese. It is evident that in its entirety this apparatus has the typical urodele form.

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PREPARATION OF MAMMALIAN SKELETONS.

By Bertram G. Smith.
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The preparation of mammalian skeletons by a person without experience usually involves a considerable loss of time and material before the precise mode of procedure adapted to secure the best results can be determined. Most text-books on the anatomy of the cat give brief general directions for preparing skeletons, but much is left to be learned by experience. It is hoped that by the use of the following detailed directions a successful preparation can be easily and quickly made at the first attempt. If carried on in the manner indicated, the work is devoid of all disagreeable features. The cat is chosen as an example, but the methods given, with slight modifications, will apply equally well to almost any other mammal.

Unless the operator is already quite familiar with the anatomy of the bones, he should consult in connection with his work the illustrations and descriptions in some good work on the anatomy of the cat, such as Jaynes' Mammalian Anatomy, Reighard and Jennings' Anatomy of the Cat, or Wilder and Gage's Anatomical Technology.

1. *Preparations required.* Each student should have for individual use a set of bones as follows:

(a) One complete skeleton with all the bones disarticulated except the hyoid, sternum, and the bones of the hand and foot of one side.

(b) One articulated skull.

There should also be provided for class use at least one natural skeleton (i. e., a skeleton prepared without disarticulating the bones, or with the bones wired together in the natural position). The cartilaginous parts should, as far as possible, be retained. In addition it is well to prepare several trunk skeletons—articulated vertebral columns with attached pelvis, ribs and their cartilages, and the sternum.

A few skeletons of young kittens should be prepared to show the incomplete development of some of the bones at this stage—particularly bones that develop from several centers, as the innominate bones, humerus, femur, etc. The skulls should be articulated to show sutures, for which purpose they serve especially well; the bones of the hand and foot, at least, should also be prepared articulated.

2. *Selection of Specimens.* For most purposes cats just old enough to be full grown are best—from one to three years old.

In kittens many of the bones are not shown in the completely developed state, and the epiphyses are likely to separate from the diaphyses; consequently such specimens are not good for general purposes. When the kittens are at least half-grown, their skulls do very well for disarticulated preparations; the bones are very easily taken apart, but are not so strong and durable as those of older specimens. The ear ossicles are well developed in kittens, and are easier to extract without injury from a young than from an old specimen.

On the other hand, cats may be too old to be desirable. Aged cats (i. e., those from 6 to 8 years old) often have exostes, or abnormal growths, on their bones. Moreover, the sutures of some portions of the skull may be entirely obliterated, making it impossible to trace them in the articulated skull, and impracticable to separate the bones. The sphenoid is especially likely to be broken in such skulls. The teeth are often missing or decayed. The stapes is brittle and held by tough ligaments. Old cats require a much longer time for boiling before the flesh can be separated from the bones.

For an articulated skeleton, a middle-aged specimen is to be preferred, the larger the better.

3. *Collecting.* The cat can be most conveniently carried to the laboratory in a sack with coarse mesh to admit air. Sometimes it will be found better to kill the cat before its transportation, as a dead cat is more convenient to carry than a live one.

4. *Killing.* Chloroform should be used if the cat is killed in the laboratory. Place the cat in an air-tight keg, jar or box no larger than is necessary, and throw in a handful of cotton saturated with 3 or 4 teaspoonfuls of chloroform; cover tightly and hold the lid down while the animal is passing through the stage of intoxication. The cat will be dead in about 15 minutes. If cats are to be killed away from the laboratory, drowning is a convenient method and free from all objections except some slight discomfort to the cat. Place the cat in a sack and hold it under water for a few minutes.

5. *Removal of Skin.* The work is more easily done soon after the animal is dead, before *rigor mortis* sets in. Place the ani-

mal on its back on a large dry-goods box or table; stretch the limbs laterally at right angles to the body and fasten in that position by cords attached to the paws by slip-knots and tied to nails driven into the box or table, one at each corner.

With a sharp scalpel make a longitudinal incision through the skin in the median line just posterior to the point of the chin. Introduce a grooved director into this cut and with the grooved side upward push it just below the skin along the median line toward the tail. The skin can now be easily divided by pushing the point of the scalpel along the groove of the director. In this manner the skin should be divided along the ventral side in the median line until the tail is reached. Then starting at the median line, divide the skin of each leg in a similar manner.

In removing the skin, lift up the cut edge with the fingers of the left hand, thus putting the superficial fasciæ on the stretch and with the scalpel held in the other hand divide the loose tissue lying in the angle between the skin and the body. Remove as much fat as possible with the skin.

In removing the skin care must be taken not to injure the hyoid and the clavicles. The clavicles are best located and removed at this time, and tied up in a piece of cheese-cloth.

After the skin has been reflected about the anus, put the skin on the stretch, dissect the rectum free from the surrounding pelvis as far as possible, and tie to prevent escape of contents of intestine. Cut the skin posterior to the ligature. This procedure will assist in the later removal of the intestine, and is much better than to tie and divide the rectum within the body cavity as is usually directed.

It will not be necessary to remove the skin from the hands, feet and the tip of the tail, as it will come off more readily after boiling.

6. *Removal of Viscera.* Beginning at the tip of the xiphisternum and extending posteriorly to the pelvis, make a longitudinal incision into the body cavity. Divide the œsophagus and trachea; avoid cutting the larynx, as the hyoid may be injured thereby. Cut the diaphragm free from its attachment to the body wall, insert the hand into the thoracic cavity, tear the contents free from the thoracic walls and pull them together with the windpipe and œsophagus backward and out through the incision in the abdomen. Separate the mesentery fastening the abdominal viscera to the dorsal body wall, free the rectum from the pelvis and pull it forward, thus removing all the viscera.

7. *The Liquid Soap Method of Cleaning the Bones.* This method, first recommended by Wilder and Gage, is best for the preparation of skeletons from fresh material. Specimens preserved in formalin or alcohol are not easily prepared by this method.

The cleaning is done by boiling the bones in a dilution of the following mixture:

Distilled or rain water, 2000 c. c.
Concentrated ammonia, 150 c. c.
Potassium nitrate, 12 g.
Hard soap, 75 g.

The advantages of this method are the rapidity of the process and freedom from the disagreeable odors attendant upon the old maceration method. The liquid soap saponifies the fat and aids in softening the connective tissue. The bones come out white and free from grease. It is especially adapted for skulls, as the teeth are much less likely to fall out than with the maceration method, and the gelatinized dental periosteum, serves as a cement, when it dries. There is no danger of blood poisoning if fresh healthy specimens are used.

Use a porcelain or granite-ware kettle to avoid discoloring the bones.

8. *Preparation of Set of Bones for Individual Use.*

(a) *Boiling and Cleaning.* After skinning the cat and removing the viscera and clavicles, cut away as much of the flesh as is convenient with a sharp knife. Remove the head, being careful not to injure the hyoid apparatus. Separate the limbs from the trunk, and sever the tail from the body at the union of the first caudal vertebra with the sacrum. Separate the vertebræ of the sacral and lumbar region from the thoracic vertebrae. Fasten together the limbs of the right side, which are to be used for articulated preparations of the hand and foot, with a string which may be allowed to hang out over the edge of the kettle, so that these bones may be easily removed for examination during the process of boiling. It is best to make disarticulated preparations of the hand and foot of the left side, since the bones of this side are illustrated and described in great detail in Jaynes' Mammalian Anatomy.

It is best to attempt only one skeleton at a time to avoid any possibility of mixing the bones. There is no loss of time after the first boiling.

Place the bones in a kettle with enough liquid soap solution in four parts water (1:4) to cover them. Boil just enough so that the skin of the paws may be readily removed with the forceps. This will usually take from 20 to 40 minutes, according to the age of the specimen. Remove the right hand and foot; the hand should have the radius and ulna attached, and the foot may be left joined to the tibia and fibula. As the bones will be too hot to handle, they may be cooled by immersion in lukewarm water. Remove the hyoid without disarticulating, by dissection with forceps and the small blade of a dull knife. Also remove the sternum entire, by cutting the lateral cartilages connecting it with the ribs. Pour off the liquid from the remaining bones and replace by an equal amount prepared in the same way. Boil again from one-half to three-quarters hour, or until the greater part of the flesh readily comes off from the bones. Meanwhile clean up the articulated hyoid, sternum, hand and foot without further boiling. This is best done with forceps and a small-bladed dull knife. Care should be taken not to injure the ligaments of the joints of the bones of the hand and foot. These will be greatly swollen, so that it will be impossible to make a neat looking preparation at the time it is cleaned; but the ligaments will shrink greatly while drying, so that the appearance of the preparation will be greatly improved. After it is dry, any remaining flesh or rough projections of cartilage may be removed with a knife.

Take the bones from the kettle and by means of the hands, a dull knife, and a nail brush, remove as much of the flesh as will come off easily. Fasten the vertebræ on a copper wire without disarticulating. Remove the ribs one by one, laying them in order on a board as removed, and fasten each set together in order with a string. Stir up the brain with a wire, taking care not to injure the projecting tentorium, and rinse out as much as is convenient with hot water. If the skull is to be prepared without disarticulating, remove the mandible at this time without separating its two rami, and clean up without further boiling. If boiled longer the mandible is likely to come apart at its symphysis. Wrap and tie each set of the remaining bones in cheesecloth and cover with a fresh solution of liquid soap. This time it is well to make the solution stronger—say one part of liquid soap to two parts water.

Boil the bones until the soft parts may readily be removed with a nail brush. This will usually take from one-half to three-quarters hour. If the skull is to be prepared without disarticulating, care should be taken not to boil it too much, as some of the bones, particularly the lachrymals, nasals and malars, also the teeth, are likely to become loose and fall out. Hence a skull intended for this purpose should be carefully watched, and after 15-30 minutes boiling it is usually advisable to remove it from the kettle and clean it up. The brain should be thoroughly removed with forceps, wire, and by rinsing with hot water. The ear cavity should be carefully cleaned with forceps and rinsed with water by means of a pipette, and the malleus and incus removed with delicate forceps. Keep a record of the side from which each set of ear bones is obtained. The stapes is best removed with a hooked pin; it is very delicate and easily lost or crushed by careless handling. Great care is required to avoid pushing it into the cavity of the internal ear; if this is done it usually cannot be recovered.

Clean up the pelvic girdle at the same time as the skull. It is well to leave the ossa innominata united at their symphysis, and further boiling is likely to separate them.

The other bones should be boiled until the work of cleaning with a brush can be carried on with the greatest ease. Time is lost by attempting to clean them too soon. The phalanges may be kept in order by placing each bone as soon as separated from the rest in a box with numbered compartments.

If the bones are not entirely free from grease boil them again, excepting the articulated preparations, in liquid soap solution 1:1 for about half an hour. Rinse them thoroughly in clean water and place them on white paper to dry in the sun. This will bleach them sufficiently. The drying should be thoroughly done.

(b) *Disarticulating Skulls.* Boil the skull, prepared as directed above, from one-half hour to an hour in liquid soap 1:1. If the specimen is young, the bones may now be separated by steady pulling with fingers or forceps. In the case of an older animal, it may be necessary to fill the cranial cavity with peas, beans or rice, force a cork into the foramen magnum, and soak the skull for a few hours. This will burst apart the dorsal sutures and the process of disarticulation may then be completed by hand. Especial care is necessary to avoid breaking the sphenoid, the ethmoid, and the maxillo-turbinals, and to avoid losing the lachrymals.

(c) *Cements for Bones and Teeth.* Teeth that become loose during boiling will often be found firmly fixed in place after the skull is dry, hence only teeth that remain loose after drying need be reset. For this purpose, use plaster of Paris. Bones that have come apart at their symphyses may be fastened together with liquid glue.

(d) *Labeling.* Assign a number to each set; this number should be placed upon each bone or articulated preparation, and upon each box, tray or vial containing bones of the set. In the case of bones too small to label, as the ear ossicles, sesamoid bones, etc., seal up the vial containing the bones; the number on the vial will then be sufficient. All very small bones should be kept in vials, to prevent loss or injury. The body vertebræ and the first four caudal vertebræ may be strung in order on a wire. The labeling of the bones with the number of the set should be done with water-proof India ink; in case it is desired to use numbers to indicate the order of arrangement of any of the bones, as the ribs, vertebræ or phalanges, these numbers may be distinguished by using red ink or Roman numerals. Vials containing ear ossicles, and boxes containing the manus or pes, should be labeled right or left.

9. *Boxes for Skeletons.* The set of boxes specified below is intended to contain a set of bones for individual use.

The outside box and trays, with covers, should be made of heavy pasteboard, covered with black cloth and lined with paper. Compartments of trays should be made of separate complete paste board boxes without covers, glued in place. The boxes and trays trays may be numbered and labeled with white ink.

The outside measurements of trays should be adhered to; measurements of compartments make no allowance for thickness of material.

(a) Box to contain trays specified below. *Inside* dimensions 31 cm, x 17.5 cm. x 12.5 cm. Telescope cover.

(b) Three trays as follows: (1) Tray to contain bones of disarticulated skull. See Fig. 1. *Outside* dimensions 30 cm. x 16.5 cm x 3 cm. The covered spaces may be used for posting notices or records of inspection.

The ear ossicles are best kept in small vials, in the same compartment with the temporal bones. The lachrymals should also be kept in a vial, and placed with some other bone. The ethmoid may be placed in a short shell vial, or wrapped in tissue paper. The vomer should be left attached to the ethmoid, the maxillo-turbinal to the maxillary.

(2) Tray to contain limb bones (except the larger bones, such as the femur, humerus, pelvic girdle, etc.). See Fig. 2. *Outside* dimensions 30 cm. x 16.5 cm. x 3 cm.

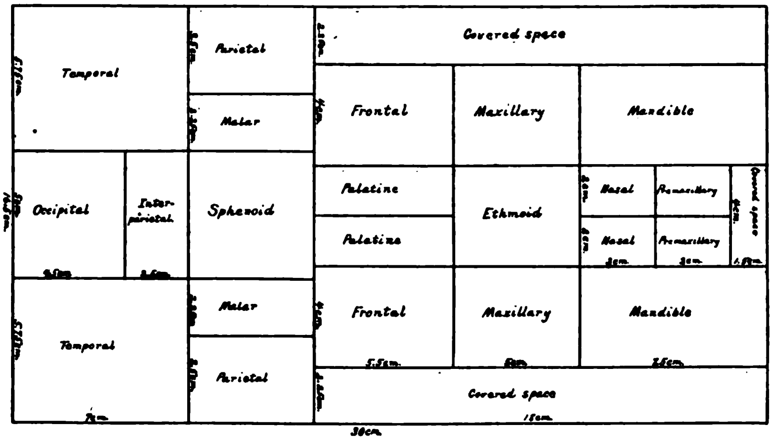

Fig. 1

(3) Tray to contain articulated skull and remaining bones. See Fig. 3. Outside dimensions 30 cm. x 16.5 cm. x 6 cm. The clavicles, chevron bones, and the small vertebræ of the tip of the tail, are best kept in vials; the articulated hyoid may be kept in a shell vial.

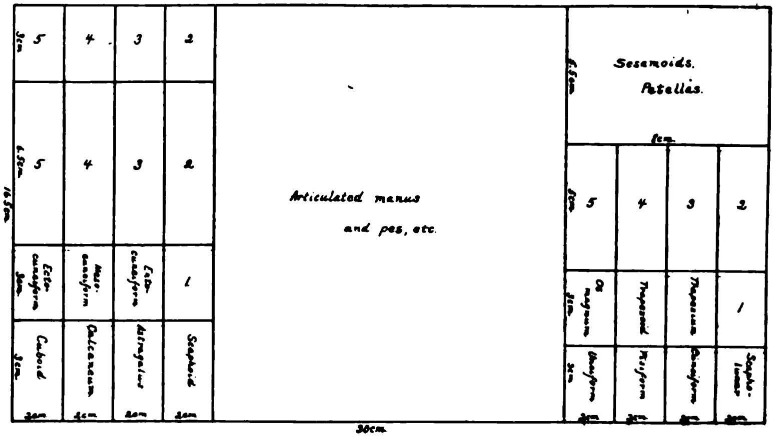

Fig. 2.

The trays should be provided with hinge covers, each consisting of two flaps attached at the longer sides of the trays.

10. *Preparation of Natural Skeletons.* A skeleton held together by the natural ligaments may be prepared by boiling for only a short time in liquid soap solution 1:4 (about one-half to three-fourths hour, depending on the age of the specimen—about as much as was recommended for articulated hands and feet.) Clean up the preparation as well as possible without injuring the ligaments, taking great care not to dislocate any of the bones by careless handling; then soak for 3 to 6 hours in a saturated solution of sodium arseniate (about 25 per cent) to poison the ligaments and protect them from insects. Then arrange the parts in a natural position and support the skeleton with wires. When the preparation is dry and the previously swollen ligaments are shrunken, finish the process of cleaning by trimming with a sharp knife.

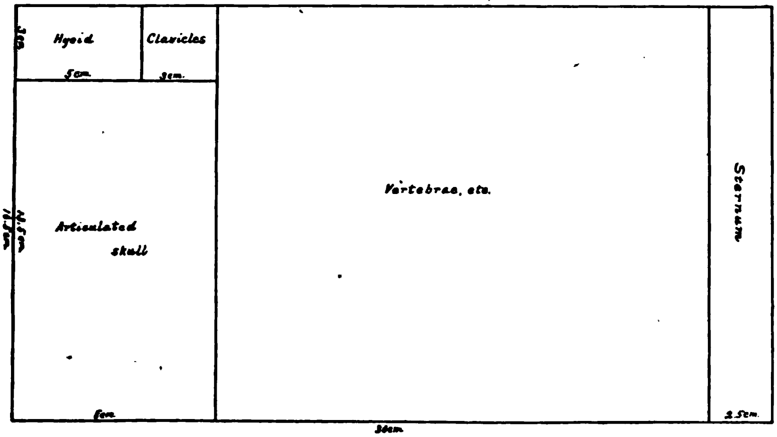

Fig. 3.

If it is desired to have the ligaments flexible, soak the preparation, after it is partly dry, for 12 to 16 hours in a mixture of saturated solution sodium arsenate 4 parts, and glycerine 1 part.

Preparations made by first disarticulating the bones of the body, thoroughly cleaning and then wiring them together, are troublesome to prepare and are best purchased from a dealer in natural history supplies.

11. *Preparation of Bones of Kittens.* The liquid soap process is best for this purpose, but the boiling should be carried little

further than for the making of a natural skeleton. Ten or fifteen minutes boiling is sometimes sufficient for a young kitten. The bones must not be boiled until the epiphyses separate from the diaphyses.

5

January, 1907.

VOLVOX FOR LABORATORY USE

BERTRAM G. SMITH

In providing a supply of Volvox for class use late in the fall, difficulty has been experienced in two respects: in keeping the material alive in the laboratory as long as desired, and in getting specimens containing sperm and ova. Inquiry reveals the fact that others have had the same trouble. I have recently been able to overcome both these difficulties, and at the suggestion of Dr. H. H. Newman have recorded the method in some brief notes.

Species.— So far as known, the only species of Volvox that has been found in the vicinity of Ann Arbor during the late autumn is *Volvox aureus* Ehrenb. It is not very abundant, and I have never found it in the sexual stage at the time it was collected. It occurs in small glacial pools containing Riccia and duckweed.

During the early spring *Volvox globator* Linn., and no other species, occurs in great abundance in the same pools that later contain *Volvox aureus*. I have occasionally found it in the sexual stage when collected.

By the latter part of June *Volvox globator* has become quite scarce, and *V. aureus* has begun to appear. During the early part of July the two species exist in the same habitats, but neither is very abundant.

Since Volvox is so widely used for laboratory work by beginning classes, the marked specific differences are matters of importance. *Volvox globator* is the form described in text-books, but the description is far from being applicable to *V. aureus*. Since the latter may at times be the only species available, it may be profitable to call attention to the differences between the two species, for it is to be suspected that *Volvox aureus* is sometimes used without its species being recognized.

Volvox globator is considerably larger than *V. aureus*; its somatic cells are more numerous and compactly arranged. The somatic cells of *globator* are angular and connected by very stout protoplasmic strands; the somatic cells of *aureus* are round when seen from the surface, and connected by very slender protoplasmic strands, difficult for students to make out under the microscope. *Volvox globator* is monœcious: in the sexual stage both sperm bundles and eggs may be found in the same colony at the same time. The number of sperm bundles in a single colony is small. *Volvox aureus* is either diœcious or monœcious proterogynous: sperm and eggs are never found together in a single colony at the same time, but the colony may contain one or the other exclusively. However, I have found daughter colonies, some of which contained bundles of sperms, others ova, within the same parent colony. The number of sperm bundles in a single colony is very large. *Sphærosira volvox* Ehrenb. is an old name for the male colony of *Volvox aureus.*

Kofoid ('99) gives the following key for the determination of the two species:

Cells about 10,000 (minimum 1,500, maximum 22,000), angular with stout connecting protoplasmic processes into which the chromatophore may enter. Diameter of colony about 700 μ (minimum 400, maximum 1,200); diameter of cell body 3–5 μ. *V. globator* L.

Cells 500–1000 (minimum 200, maximum 4,400); rounded, with slender connecting protoplasmic processes into which the chromatophore does not enter. Diameter of colony 170–180 μ; diameter of cell body 5–80 μ. *Volvox aureus* Ehrenb.

Klein ('99) gives illustrations of the general appearance of the two species, including reproductive stages. Meyer ('96) gives details of cell structure, with illustrations.

Volvox globator is probably a better form for laboratory work than *V. aureus*, and can be obtained in greater abundance if secured early in the spring. Hence if Volvox is to be studied in the fall by large classes it is well to preserve this species in 4% formalin in the spring; for many purposes the preserved material, if not kept too long, is as good as the living. The study of preserved specimens of *V. globator* can then be supplemented by the living *V. aureus.*

Keeping Volvox alive in the Laboratory.— Terry (:06) in experimenting on the galvanotropism of Volvox met with the usual difficulty in keeping it alive in the laboratory, and concluded that the organisms died of insufficient nourishment caused by improper food supply and poor light, but found it impossible to regulate either so that they would live for more than four days. My own experience has convinced me that in our laboratory one of the principal causes of the death of the organism has been injurious mineral substances in the tap water in which the specimens were kept.

In collecting Volvox for our laboratory it has been customary to bring in considerable quantities of vegetable material (duckweed, Riccia, etc.) from the ponds in which Volvox occurred, along with a little water, and place this material in shallow glass dishes filled with tap water. As fast as the organisms gathered on the lighted side of the dish they were picked off and removed to a dish of clean tap water, it being deemed unsafe to leave them in the original dish exposed to the attacks of crustaceans. Suspecting that deleterious substances in the tap water, as well as the lack of a proper food supply, caused the death of the organisms, I made an attempt to reproduce the natural conditions. Water containing Volvox was brought in in considerable quantities, together with a small amount of the vegetable material, and placed in shallow glass dishes without the addition of tap water. The dishes were placed near windows and covered with glass plates to prevent loss of water by evaporation and to keep out bacteria and fungi, except that when exposed to direct sunlight it was found advisable to leave room for circulation of air between the cover and the dish to prevent a rise of temperature beyond the optimum. The water was not changed at any time during the course of the experiment. In the majority of the aquaria thus prepared, Volvox flourished for several weeks; in the fall of 1905, *Volvox aureus* was kept alive in several aquaria for from four to eight weeks; less success was attained with *Volvox globator* in the spring, but it was kept alive in most cases for about two weeks.

It was noted that *Volvox globator* clusters about decaying insect larvæ, perhaps on account of the presence of carbon dioxide. A moderate amount of decaying plant or animal material in the water seems to be one of the essential conditions for its existence. In its natural environment, Volvox is often found in decidedly stagnant water.

Temperature is an important factor to be considered in caring for Volvox. It is more difficult to keep the material alive in the laboratory during warm weather because, in exposing the dishes to sunlight, the water is likely to become warmer than that of the ponds in which Volvox lives. This difficulty might be overcome by placing the dishes where they will be partly immersed in the running water of a shallow aquarium, and at the same time receive an abundance of sunlight.

In case organisms that feed upon Volvox are too abundant, the latter may be freed from its enemies by removing it with a pipette when clustered at the lighted side of the dish, to a dish of pond water strained through bolting cloth to remove crustaceans, etc.

Obtaining Volvox in the Sexual Stage.— Both in the fall and in the spring, material in the sexual stage was obtained in abundance merely by keeping the organisms alive in the laboratory. Several aquaria should be set up, and in some of them, specimens in the sexual stage will usually be found in the course of one or two weeks. It was noted by Dr. H. H. Newman that they often remain hidden in the ooze at the bottom of the dish. When they reach the sexual stage they seem to become less motile and consequently drop to the bottom. This is especially true of sperm colonies in *V. aureus.*

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THE BREEDING HABITS OF *AMBLYSTOMA PUNCTATUM* LINN.

BY

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THE BREEDING HABITS OF *AMBLYSTOMA PUNCTATUM* LINN[1]

BERTRAM G. SMITH

On April 9, 1906, in some small ponds in a wood in the vicinity of Ann Arbor, Michigan, Prof. Jacob Reighard discovered what he surmised to be spermatophores of Amblystoma. At his suggestion and under his direction, I undertook the identification and study of these structures.

I. Observations

A. **The Spermatophores.** The spermatophores look like bunches or tufts of snowy-white fungus growing on leaves, twigs, or stalks of grass lying on the bottom of the pond. They invariably occur on a horizontal surface, and are never attached to an erect twig or stalk as is often the case with the eggs of Amblystoma. They are found in water from 6 to 10 inches deep, and 5 to 10 feet from the shore. The spermatophores usually occur in groups of about 40 or 50, but the number is extremely variable, ranging from 1 to 100. Isolated spermatophores are rarely found, though a single one is conspicuous enough to be readily discovered. The spermatophores of each group are scattered over an area of rather more than one square foot. Along the shores of an elliptical pond about 125 feet in length, 25 groups of spermatophores were counted; they were less numerous in three other ponds examined.

The spermatophores (Fig. 1.) resemble those of *Triton* (*Diemyctylus*) *viridescens* as described by Jordan ('91 and '93) rather than the more complicated structures produced by some European forms (Zeller, '05). Each consists of a base and a stalk of clear gelatinous material almost invisible in the water, having the general form of the stump of a tree; this structure is surmounted by a slightly broader cap or tuft of snowy-white felt-like material consisting of spermatozoa with no visible matrix. The material constituting the base must be strongly adhesive when fresh, for the spermatophore is firmly attached to the object on which it is deposited. The cap is usually hemispherical in form, with the convex surface upward; but the material of which it consists often runs down the side of the stalk, or is found projecting in downy tufts like the cotton from an open cotton-boll. In many cases the caps have a frayed appearance, as if they had been disturbed; in occasional specimens the cap of spermatozoa is partly or almost wholly absent. The appearance in the latter case is like that of a spermatophore of *Triton viridescens* from which I have seen the ball of spermatozoa taken up into the cloaca of a female. The dimensions of the complete spermatophore are about as follows:

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[1] Contributions from the Zoological Laboratory of the University of Michigan, No.

Height....................	6–8 mm.
Breadth of base............	6–8 mm.
Diameter of stalk near top...	2.5–3 mm.
" " cap............	3–4 mm.

As compared with some spermatophores of *Triton viridescens* obtained from specimens in captivity, these under discussion are slightly taller, with a smaller base and a stalk of much larger diameter, surmounted by a larger mass of spermatozoa. The spermatophore of *Triton viridescens* has a broad flattened base from the center of which rises a distinctly conical stalk tapering to a very slender spine, at the top of which is attached a small ball of spermatozoa; the spermatophores attributed to Amblystoma are more massive and more nearly cylindrical.

When found on April 9 and 10 the spermatophores were all in good condition, with some slight differences in the freshness of their appearance. In two or three days they became infested with fungus, disintegrated quite rapidly, and in a week very few few of them could be found. Had new ones been deposited in the interval, they could readily have been distinguished from the old ones; but no more spermatophores were deposited. Hence it is scarcely possible that the period during which spermatophores are deposited lasts longer than two or three days.

The spermatophores shown in the figure had been attacked by fungus and were beginning to disintegrate when photographed. The base is therefore no longer clear, but on the contrary the whole spermatophore appears white.

Identification. In order to identify the spermatophores, search was made for the parent animals. This resulted in the capture on April 11, of three specimens of *Amblystoma punctatum* Linn. which were found embedded in rotten wood under a stump at the edge of the water of one of the ponds where the spermatophores were numerous. From two of these specimens a few drops of seminal fluid, containing an abundance of spermatozoa, were obtained by stripping; from the third, which proved to be a female, comparatively few spermatozoa were obtained. The spermatozoa were mounted, stained, and compared with some taken from spermatophores and similarly treated. In structure, size, and staining reactions the two were identical.

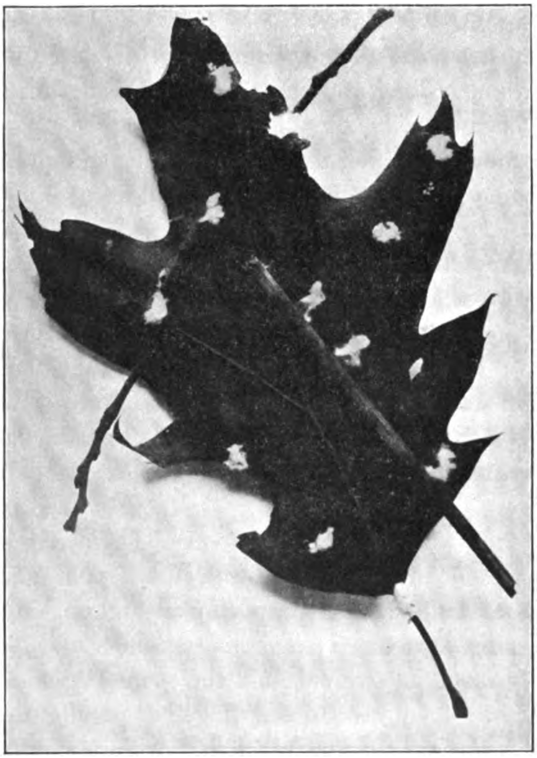

FIG. 1.— Spermatophores of *Amblystoma punctatum*. Two-thirds natural size, linear reduction.

Another species, *A. tigrinum* Green, also occurs in the vicinity of Ann Arbor, and a single example was taken on April 9, in a field several hundred yards distant from the nearest pond where spermatophores were found; but the eggs of the two species are easily distinguishable, and in the case of *A. punctatum* were identified by means of eggs laid in the laboratory. With the exception of one bunch of eggs of *A. tigrinum*, all the eggs found in the pond where spermatophores were observed, were those of *A. punctatum*. With the single exception above noted, the two species have not been known to breed in the same ponds in the vicinity of Ann Arbor.

B. **The Spermatozoa.** The spermatozoon of *Amblystoma punctatum* is extremely long and slender. The head stains well with Delafields' haemotoxylin, the middle-piece less deeply. The tail-piece is bordered on one side by a very delicate undulating membrane. Some of the dimensions are as follows:

Length of acrosome	20 μ
" " head	106 μ
" " middle-piece	14 μ
" " tail-piece	480 μ
Total length	620 μ

The spermatozoon resembles in size and form that of *Triton viridescens*, with which it was compared, but the latter has a middle-piece twice as long, and a more conspicuous undulating membrane.

As compared with the spermatozoon of *Crytobranchus allegheniensis* (Smith '06) the sperm of *Amblystoma punctatum* is nearly three times as long, with a proportionally much longer middle-piece; the entire structure is much more slender and thread-like.

In freshly mounted seminal fluid the spermatozoa were seen in active motion. They tend to cling together parallel to each other to form bundles or ringlets, revolving with a circular motion; when so clustered they retain their vitality much longer than when separated. In a dying spermatozoon, long after the shaft has ceased to move, the activity of the undulating membrane continues. It gradually becomes slower until with a high magnification it is possible to follow a trough or a crest without interruption or change of form across the entire field of the microscope. The undulating membrane does not wind about the shaft as in Cryptobranchus, but continues on one side of it. When dead, the sperms are usually found much convoluted, indicating a greater degree of flexibility than is the case with stouter spermatozoa like those of Cryptobranchus.

Experiments were performed to determine the length of time the spermatophores would retain their vitality in water, hence the interval within which they would have to be taken up by the female. In all the spermatophores examined the spermatozoa were motionless; but since the examination was not made until the evening of April 10, probably the spermatophores had been in the water for many hours. The effect of the cloacal secretion of the living female was then tried, to see if it would revive these spermatozoa; no such result was produced. Freshly obtained seminal fluid mounted in water retained its vitality for many hours; but as this experiment was not performed until April 18, only a small amount of seminal fluid could be obtained, and in this the sperms were not in a vigorous condition. If fresh seminal fluid were taken in the proper season and mounted in quantities to correspond with that deposited in a spermatophore, it might retain its vitality much longer. The viscous liquid in which the spermatozoa occur does not readily mix with water.

A freshly deposited spermatophore of *Triton viridescens* was obtained and kept in water; from time to time small portions of the ball of spermatozoa were teased apart and examined under the microscope. Eleven hours from the time the spermatophore was deposited, many active spermatozoa were found; an hour later all were motionless. Probably in an undisturbed spermatophore their vitality would be retained longer than twelve hours.

C. **The Eggs**. Those of *A. punctatum* have been described and figured by Clark ('80). The eggs, with their individual gelatinous envelopes, occur in compact bunches, surrounded by a very thick jelly mass. The entire structure is usually of an oval shape, often nearly as large as one's fist. The eggs of *A. tigrinum* are more loosely aggregated in a thinner jelly mass, and the cluster resembles a bunch of grapes. The clusters of eggs of *A. punctatum* are as a rule larger than those of *A. tigrinum,* and the number of eggs in a bunch is usually greater.

At the time of the discovery of the spermatophores, very few bunches of eggs could be found. The number steadily increased for a week; at the end of that time eggs were found in early segmentation stages, showing that they had been quite recently laid. The egg-laying season follows immediately after the deposition of spermatophores, and lasts six or seven days. Nearly every bunch of eggs found on April 10 was close to a group of spermatophores.

On April 16, in the pond where 25 groups of spermatophores had been counted nearly a week before, about 55 bunches of eggs were found. Of these, many bunches were deposited in groups of two to four, probably by the same female. The number of aggregations of eggs very nearly equalled the number of groups of spermatophores.

D. **The Adults.** *Secondary Sexual Characteristics.* During the breeding season, at least, the cloacal region of the male is quite prominent; that of the single female examined was much less swollen, and the orifice was smaller. The cloaca of the male is lined with fine parallel papillated ridges, extending inward for a few millimeters; between these ridges are deep grooves, lined with cilia whose beat is outward. These ridges and grooves were not found in the single female examined. According to Kingsbury ('95) the female Amblystoma, as well as the male, has cilia in the cloaca but the tract is less extensive. The urogenital sinus of the male is larger than that of the female, probably to hold a considerable supply of seminal fluid preliminary to the deposition of a spermatophore. No secondary sexual characters to indicate the clasping of the female by the male were found.

II. DISCUSSION.

Andrews ('97) described the structure and distribution of some spermatophores which he attributed to *Amblystoma punctatum*, but without positive identification. He states that these spermatophores were more slender and higher than those of *Triton viridescens*, and were distributed, at intervals of a few inches, in lines of several to a dozen. I find it difficult to reconcile his account with my own observations.[1]

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[1] Professor Andrews, to whom the manuscript of this paper was submitted, writes,—"The spermatophores vary in size, arrangement and form here (about Baltimore) in different years and ponds; and I think your comparison with a tree stump a good one to indicate their common form. I judge the discrepancy in our account to be one of words rather than of observations. I am convinced from your photograph and account that you have described the same spermatophores that I did, and I judge both observations — despite some differences in descriptions — refer to Amblystoma punctatum."

On account of the late season at which my investigation was begun, no direct observations of the process of fertilization were possible. Clark ('79) says of some specimens of *A. punctatum* in confinement: "The males showed no inclination to clasp the females, but quietly deposited quite large masses of an apparently rather thick liquid, opaque white, on the bottom of the dish in which they were kept. Upon examination this was found to consist of spermatozoa moving actively in a liquid." The manner in which the spermatozoa reached the eggs was not observed.

Fertilization is undoubtedly internal. Of this the evidence adduced by Kingsbury ('95), and the presence of spermatozoa in the cloaca of the female as described above, furnish sufficient proof. It remains to consider how the transfer of spermatozoa is effected by the spermatophores.

The number of spermatophores is evidently very much greater than the number of females; and unless there exists an enormous disproportion between the sexes, each male must deposit a large number of spermatophores. Their abundance and the manner of their distribution, render it a very easy matter for the female to find enough of them for purposes of fertilization. In some portions of the pond it would seem scarcely possible for a female to move about in the water for any length of time without brushing against some of these spermatophores; hence there is the possibility of finding them by chance contact.

In the cases of those Urodela in which, as in *Triton viridescens* (Jordan '91 and '93; Hilton '02) and Axolotl (Gasco '81) the number of spermatophores deposited by a single male is small, particular safeguards are needed in order to facilitate their delivery to the cloaca of the female. In these forms the physiological necessity which requires the co-operation of the female in order that spermatophores may be deposited insures the presence of the female at the right time; subsequent reactions safeguard the reception of at least one of these spermatophores by the female cloaca. In *Triton viridescens,* according to my own observations, in some cases the female seems to make a definite attempt to get the spermatophore. The complicated behavior of the adults in these cases finds its biological significance not only in the increasing certainty of the process, but in a corresponding economy in the number of spermatophores that must be deposited. With *Amblystoma punctatum,* on account of the very large number of spermatophores, there is the probability of a simpler mode of behavior, and the spermatophores may be found largely by chance. The wastefulness of the method is obvious. In Amblystoma as in Axolotl there is evidently no clasping of the female by the male, such as occurs in Triton.

The result of the experimental work on the vitality of the seminal fluid in water indicates that the spermatophore is not necessarily taken up by the female immediately after it is deposited; probably it is capable of effecting fertilization after exposure to the water for many hours.

On account of the shortness of the breeding season, the spermatozoa can be retained in the cloaca of the female for only a few days at most, before fertilization is effected. The position with respect to the spermatophores, of the earlier eggs found, suggests that in some cases the eggs are deposited immediately after the spermatophores are picked up.

The extreme flexibility of the sperm is doubtless correlated with the process of internal fertilization. In Cryptobranchus, in which fertilization is external (Smith '07), the egg envelopes must be penetrated after a brief exposure to the hardening effect of the water, and a much more rigid spermatozoon is required.

In the evolution of terrestrial from aquatic vertebrate life, a transition from external to internal fertilization takes place. External fertilization is not adapted to terrestrial conditions, hence in the land-living vertebrates it occurs only in some of the forms that revert to the water during the breeding season — *i. e.* in the Amphibia. Internal fertilization is an adaptation to terrestrial life in the sense that it is a condition antecedent to that life, not a result brought about by it; it may occur in purely aquatic vertebrates, as in the Elasmobranchs and a few Teleosts. Internal fertilization by means of spermatophores is a method still adapted to aquatic rather than to terrestrial conditions. It is a method intermediate between external fertilization on the one hand and internal fertilization without spermatophores on the other. Viewed in the light of the habits of the higher vertebrates, the occurrence of any method of internal fertilization in a form that breeds in the water represents an advance upon the habit of external fertilization, and a stage in the evolution of habits that are to make possible the invasion and permanent occupation of the land.

Internal fertilization also finds a biological significance in the fact that in the course of its development there is gradually effected an economy in the amount of seminal fluid required for fertilization. This factor may account for the persistent development of the habit under aquatic conditions, where external fertilization is still possible; the incidental result is a preparation for terrestrial life.

In existing Amphibia we may find illustrations of various stages in this evolution of the breeding habits correlated with a transition from the water to the land. In Cryptobranchus, one of the lowest of the Urodela, leading an aquatic life and showing only in its methods of respiration and locomotion an advance toward terrestrial conditions, external fertilization takes place. This is evidently the primitive condition for the Urodela. In Amblystoma, a urodele living partly upon the land but returning to the water to breed, we see developed the peculiar habit of fertilization by means of spermatophores — a mode of internal fertilization favored by aquatic conditions. In *Triton viridescens* an economy of seminal fluid through a reduction in the number of spermatophores is made possible by definite reactions on the part of the adults, which insure fertilization. In the urodeles *Megapterna montana* Savi., *Molge aspera* Dugès and *Glossoliga Hagenmulleri* Lataste, according to Bedriaga ('82 and '95) the male emits spermatophores while still clasping the female; in *Triton torosus* Esch. (Ritter '99) it is probable that a very similar process occurs; in none of these cases, with the possible exception of *Molge aspera*, is there direct cloacal contact. Finally in the Apoda (the Sarasins '87–'93; Brauer '97) we find the establishment of a method of internal fertilization by direct cloacal contact, thus fulfilling the requirements for continuous residence upon the land.

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THE LIFE HISTORY AND HABITS OF CRYPTOBRANCHUS ALLEGHENIENSIS

BERTRAM G. SMITH

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THE LIFE HISTORY AND HABITS OF CRYPTOBRANCHUS ALLEGHENIENSIS.[1]

BERTRAM G. SMITH.

(Contributions from the Zoölogical Laboratory of the University of Michigan, No. 109.)

I. HISTORICAL.

In spite of persistent attempts to work out the natural history of the giant salamander, *Cryptobranchus allegheniensis*, the habits, particularly the breeding habits, have remained little known. Very little has been learned about the development of the eggs, and the larvæ have not been described.

The first account is that of Townsend ('82) and consists of brief notes on the behavior and feeding habits, with a general description of some eggs deposited in August. McGregor ('97) described very briefly an embryo 16 mm. in length. Reese ('03) discussed principally the size, coloration, movements and feeding habits of the adults, and recorded his persistent attempts to obtain embryological material. In a later paper ('04), he gave the first accurate description of the unfertilized eggs.

Recently ('06[2]) I published a detailed description of the eggs and spermatozoa, and an account of the early stages of the development, with some incomplete observations on the breeding habits. The material for this paper was secured during the fall of 1905.

During the months of August and September, 1906, in northwestern Pennsylvania, I devoted my entire time to the study of *Cryptobranchus*, with the object of securing a knowledge of its habits, and material for the study of its development. The purpose of the present paper is to record the results of this work so far as they contribute to a general account of the natural history of the animal.

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[1] The writer is indebted to the Elizabeth Thompson Science Fund for Grant No. 130.

II. The Adults.

A. *Habits Not Peculiar to the Breeding Season.*

Habitat. — *Cryptobranchus* was found most abundantly in a large creek tributary to the Allegheny River, and the most favorable locality extended from its confluence with the Allegheny five or six miles up the stream.

The stream has a rather rapid descent, and a gravelly or rocky bottom. Shallow and rocky rapids make up the greater portion of its course, alternating with areas of deeper and more quiet water. It is in the former situations (see Fig. 1) that *Cryptobranchus* abounds, lying concealed for the greater part of the time in crevices or caverns under large rocks in the stream bed. It is seldom that more than one individual is found under a rock, hence its life is in general a solitary one.

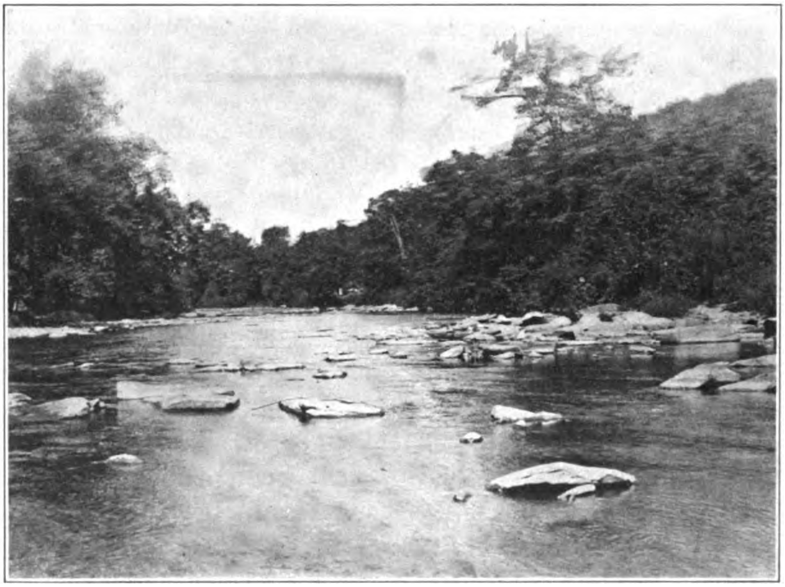


Fig. 1. Typical habitat of *Cryptobranchus alleghenicnsis.*

So far as my observations extend, *Cryptobranchus* comes forth but seldom in the day time, except during the breeding season. At night they venture abroad, perhaps in search of food; and fishermen who have speared by torchlight tell marvellous tales of the number of these creatures they have seen, usually lying quiet on the bottom when observed. According to Townsend ('82), in the early summer, when the water is clear, hellbenders are often seen on the bottom in considerable numbers; in August he found them only under rocks. Presumably his observations were made by daylight.

The cavity or cavern used as a dwelling-place has the rock for its roof and the gravelly bed of the stream for its floor. In perhaps the majority of cases, ready-made caverns are chosen as homes, and these are reached by a natural opening. But the cavity often bears evidence of having been in part hollowed out by the animal, and is sometimes reached by a single burrow-like entrance, with a little heap of freshly excavated gravel at its mouth, on the downstream side of the rock. I have occasionally seen the front limbs used to scratch and push away sand and gravel, while the animal was forcing its way under a rock; but the burrowing habit is only slightly developed. It is interesting to compare this beginning of the burrowing habit in *Cryptobranchus* with its marked development in the closely related but more terrestrial *Amphiuma.*

There is a striking similarity between the habitat of the American *Cryptobranchus* and that of the giant salamander of Japan (*C. japonicus* v. d. Hoeven, or *Megalobatrachus maximus* Schlegel), as described by Ishikawa ('04); but in the case of the latter the burrowing habit appears to be better developed.

Cryptobranchus does not thrive except in cool and shallow running water. When a specimen is placed in a tank of quiet water, it soon shows great uneasiness, swimming restlessly about as if seeking means of escape, and coming frequently to the surface for air. In a short time, as stated by Reese ('03), the stomach contents are regurgitated. So long as kept in such an unfavorable situation, it refuses food.

Size. — Although during the season I handled more than a hundred specimens, the largest adults of both sexes measured only 53 cm. in length. Townsend ('82) records the capture of some specimens 22 inches (56 cm.) long. The longest specimens obtained by Reese ('04) measured 55 cm. By far the greater number of specimens captured by me were much smaller than the extreme size given; specimens of about 35–40 cm. were apparently most numerous. The smallest sexually mature males measured about 33 cm.; females 35 cm.

Form. — The general form of the body (see Fig. 2) is such as to adapt it to bottom life, and to shallow crevices under rocks. The flattened head is wedge-shaped in a horizontal plane, enabling the animal to force itself into very shallow crevices. The fore limbs are adapted for ordinary locomotion, for climbing over rocks, and for use to a slight extent in burrowing.

As compared with the young, the adult is distinguished by the general looseness and wrinkling of the skin at the sides of the body, forming folds which become more prominent in adult life, and by the flaps of skin on the posterior sides of the limbs. During locomotion these folds and flaps undulate in the water, contributing to the uncouth appearance of the animal; they hang limp when the living specimen is taken out of the water, but become stiffened in preserved specimens.

Coloration. — Young adults vary but little in color or color pattern. The ground color is a dull brown, with conspicuous black spots and less conspicuous yellow spots, irregular in form and distribution, scattered over the dorsal and lateral surfaces. Since the coloration of the young adult is practically the same as that of immature specimens from 16 cm. upward, Fig. 14, from a specimen 26 cm. long, will serve to represent this stage, in which the spots are more conspicuous than in the young larvæ or the more mature adults. In older specimens (see Figs. 2–4) the general color effect may vary in two ways: it may become either greenish brown, or decidedly reddish brown. As stated by Reese ('05), these variations in color occur in both sexes.

The coloration of the dorsal surface is protective, closely resembling the gravelly bottom on which *Cryptobranchus* lives. The spots contribute largely to this general effect. When the stone under which a hellbender lies is overturned, the animal sometimes remains perfectly motionless, as if instinctively relying upon its coloration for protection. On account of its size, and the possession of various means of defense, it is probable that *Crypotobranchus* has few enemies, and its coloration is of value to it principally as a means of concealment while lying in wait for prey. The head, especially, resembles a flat stone covered with silt; when the animal is lying in a favorite position, under a rock with only the head exposed, it is extremely difficult to recognize it.

Locomotion. — The ordinary method of locomotion is by crawling slowly along the bottom. The limbs extend laterally and give little support, so that the abdomen lies in contact with the bottom. The order of movement of the limbs is the same as that of a trotting horse; the limbs which move together in the same direction are associated in diagonal pairs (Marey, '79). The pressure of the body on the ground is always diagonal; the body is supported and moved forward by a right fore foot and a left hind foot at the same time the other two limbs swing free from the ground and reach forward. The result is that the limbs on one side of the body are brought nearest together while those on the other side are extended furthest apart. The movement may be illustrated by two persons paddling a canoe, each with a long double-bladed paddle grasped by the middle; let one execute a stroke on the left hand at the same time the other makes the same movement on the right; let this performance be alternated with a similar one in which each person makes a stroke on the side opposite from that previously taken by him.

Cryptobranchus is also a good swimmer. In swimming the body undulates in a horizontal plane, like that of an eel, and the tail shares in this motion; in the most rapid swimming the motion of the tail is very vigorous, and is the principal means of propulsion. The legs, however, are of considerable use in swimming, both to propel and to guide the body and to preserve its equilibrium; during swimming at a moderate rate of speed the legs are the main propelling organs. The legs preserve their usual order of movement during swimming. Swimming is, then, effected by the combined motion of body, limbs and tail, the limbs playing a more important part in propulsion than the lateral fins of most fishes. Hence the swimming movements, as compared with those of fishes, present an advance toward those of most of the higher animals, which swim by the use of the limbs. *Cryptobranchus* often swims close to the bottom, so that locomotion is effected by a combination of crawling and swimming movements.

Breathing Movements. — The process of breathing by means of the lungs has been briefly described by Reese ('03); the following details may be added. In rising for air the anterior end of the body is stretched slowly in an oblique direction upward;

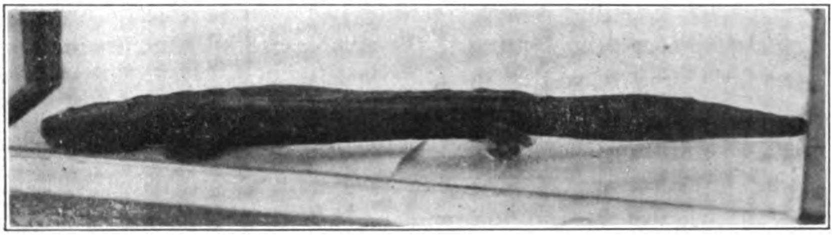


FIG. 2.

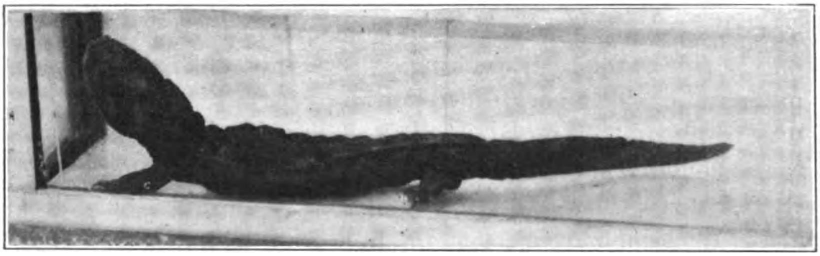


FIG. 3.

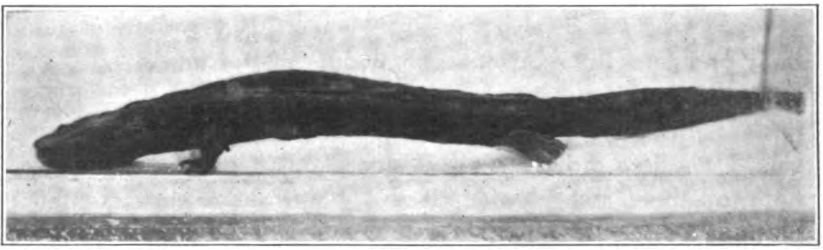


FIG. 4.

FIGS. 2–4. An adult female *Cryptobranchus alleghenienssis*, 53 cm. in length. This specimen, full grown when captured, has been kept for seven years in an aquarium in the Zoölogical Laboratory of the University of Michigan. It is a trifle slender as compared with most newly-captured specimens. The white spots on the back are due to a recent growth of *Saprolegnia*.

FIG. 2. Normal resting position.

FIG. 3. Attitude when about to rise for air.

FIG. 4. Attitude when the lungs are fully inflated.

in shallow water the posterior end often remains under a rock. Fig. 3 shows an early stage of this movement. The air taken in through the nares at the instant the tip of the snout reaches the surface is probably immediately afterwards mixed with respired air expelled from the lungs; then the greater part of the mixed air is forced back into the lungs by a swallowing movement. The surplus air escapes through the mouth or gill slits as the animal sinks to the bottom; but enough air is forced into the lungs to elevate this portion of the body, giving the back an arched appearance (see Fig. 4). A little later, more of this air is expelled in order to enable the animal to resume its normal resting position (Fig. 2), and to prepare it for another inspiration.

According to Whipple ('06), the ypsiloid apparatus (see also Smith, '06[1]) of many Urodeles subserves a hydrostatic function in changing the angle of inclination of the body in a vertical plane by pressing upon the posterior part of the expanded lungs and forcing the air forward. It is suggested that this may be the case with *Cryptobranchus*. But the animal makes active paddling movements with its forelimbs in rising to the surface for air; moreover when the lungs of a freshly killed specimen are inflated to the limit by means of a blow-pipe, their slender tips do not reach to the ypsiloid apparatus. Hence in the case of *Cryptobranchus* it seems impossible that the apparatus should have a hydrostatic function, excepting the slight effect produced by pressing the abdominal viscera forward.

Specimens in swiftly flowing water in cool weather rarely come to the surface to breathe. I have watched specimens that have remained motionless on the bottom for hours. On one occasion a dozen hellbenders in a covered creek aquarium built of wire netting to allow a constant current of water, were submerged by high water for two days without apparent injury. In these cases cutaneous respiration is probably sufficient; this may be aided by pharyngeal respiration, but in specimens confined in aquaria, having access to air, I have been unable to detect any current of water flowing in at the mouth and out at the gills, such as occurs occasionally in a resting *Necturus*. According to Gage ('91) the oral epithelium of *Cryptobranchus* is stratified and non-ciliated, as is usually the case with Amphibia whose respiration is mostly aquatic. The branchial slit is guarded at its pharyngeal opening by two valve-like flaps, in position and appearance like degenerate gills, which prevent the entrance of water from without. Their respiratory service, owing to their small size, is probably very insignificant.

Feeding Habits. — Examination of stomach contents shows that crayfishes form the principal food of *Cryptobranchus*; fishes are eaten only occasionally. Out of a dozen specimens examined in August, nine were found to contain crayfishes; only three contained fishes. *Cryptobranchus* sometimes takes a hook baited with earthworms, if cast near it. In captivity it will eat almost any small moving animal, or pieces of meat moved along a little to one side of the head. A specimen kept in an aquarium in the Zoölogical Laboratory of the University of Michigan is reported to have eaten frogs on several occasions, and once a toad. One of my newly-captured specimens seized a young *Necturus* about eight inches long, but soon released it. The adult *Cryptobranchus* will eat the eggs or larvæ of its own kind. Specimens kept in a creek aquarium in running water do not refuse food, even immediately after their capture.

Like many other amphibians, *Cryptobranchus* eats its own shed epidermis. The epidermis usually comes off in ragged pieces, giving the animal a tattered and uncouth appearance. The epidermis from the feet comes off like the fingers of a glove. The mouth is sometimes used to aid in the removal of the fragments. The practice of eating the shed epidermis is probably quite common amongst the Urodeles. I have observed a *Triton* (*Diemvctylus*) *viridescens* pulling off and eating the glove-like epidermis from the foot of another specimen. Ritter ('97) states that the shed epidermis of *Triton* (*Diemyctylus*) *torosus* forms an important article of diet for the animal.

Cryptobranchus apparently takes no notice of its prey until the latter approaches within a very few inches of its mouth and a little to one side, then seizes it by a remarkably quick sidewise movement. Perhaps the striking distance corresponds to the distance at which it can see clearly, or it may be the result of experience in catching wary prey. The enormous size of the mouth may compensate for the rather deficient eyesight (see

Reese, '05); it also makes possible the capture of rather large animals as food. No attention is ordinarily paid to pieces of meat unless they are in motion, or attract notice by actual contact. The rocks under which *Cryptobranchus* lurks also afford cover for its prey, hence a food supply may be obtained without leaving shelter.

Although I have handled the animals very often and without caution, I have never been bitten by them. A specimen confined in the Zoölogical Laboratory of the University of Michigan is said to have tried to bite several persons; this happened only after it had become accustomed to its surroundings and to the presence of people about it; probably it was only the usual feeding reaction, which had previously been inhibited by discomfort or fear.

Slime Production.—The surface of the body is at all times slimy enough to be extremely slippery, making the animal difficult to hold except when grasped by the neck. This constant secretion is of value to the animal in protecting the skin from abrasion in gliding over a rough rock, and perhaps also from parasites. But when a hellbender is injured or seized and forcibly held, an abundant flow of sticky, whitish, gelatinous slime, resembling the "milk" from the broken end of a milkweed stem, breaks out over the entire body or sometimes only from the tail. This slime causes a smarting sensation when it comes in contact with the freshly-cut surface of the skin.

Profuse slime production is at least indirectly a result of mechanical stimulation, though probably under the control of the nervous system, and associated with fear.

Reactions to Mechanical Stimuli.—The adult *Cryptobranchus* is strongly thigmotactic, seeking crevices under rocks or an angle of the aquarium in which it is confined—a result not entirely due to a negative reaction to light. It may even force its way under a stone of considerable size where no crevice exists, lifting the stone bodily in the water.

A shock or jar in the water sometimes causes a quick jerking movement, and withdrawal under a convenient cover; but the adult reacts far less readily to a shock than the larva.

When forcibly held, the animal wriggles actively, and sometimes makes a rather loud crackling sound, like that produced by boys who snap the joints of their fingers. The noise seems to come from the articulations of the vertebræ.

When tightly squeezed about the neck, the hellbender sometimes utters a shrill cry, perhaps due to the involuntary expulsion of air from the lungs.

Reaction to Light. — As previously stated, *Cryptobranchus* avoids the light. Like its thigmotactic response, this reaction is an important factor in its customary mode of life, since it usually seeks by day the cover of rocks where, concealed from possible enemies, it lies in wait for prey. Specimens confined in the laboratory are nocturnal in their activities, and during the day seek the darkest corner of the aquarium. According to Reese ('06), the tail is much more sensitive to light than the head or the middle part of the body. This may partly account for the favorite position of the animal — lying under a rock with only the fore part of the head exposed.

B. *Breeding Habits.*

Sexual Differences. — The adult male may be recognized (Reese, '04) by the presence of a swollen glandular ring or ridge of tissue about the opening of the cloaca. This is especially prominent in the breeding season, but may be recognized before the breeding season begins.

Females in the breeding season may be distinguished by the swollen appearance of the abdomen. As the egg-laying season approaches the eggs collect in the uteri, and when a ripe female is held in a vertical position with head uppermost, the lower portion of the abdomen sags and bulges out.

Females were found to be quite scarce or perhaps inaccessible as compared with the males. The ratio in those captured was about 1: 8.

Breeding Season. — As the time of the beginning of the breeding season was not accurately known, on August 11 I examined the reproductive tract of a newly captured male 34 cm. in length. The vasa deferentia were found to be contracted, about 2 mm. in diameter, and almost empty, containing only a very few spermatozoa, and these at the upper end. The spermatozoa were immotile. In the testes very few spermatozoa were mature.

On August 14 a large female was examined. The ovaries contained several hundred eggs almost fully developed, besides many smaller ones. The oviducts were empty.

Observations of this kind were continued throughout the month. On August 24 a female was found in which a few eggs had descended into the oviducts and become surrounded by a gelatinous envelope. This specimen was somewhat exceptional, as in others examined up to September 1 the eggs had not left the ovaries. Evidently there is considerable individual variation in the time when females ripen. On September 1 the first nest of eggs was found; these were in the first and second cleavage stages, hence had probably been deposited about 24 hours before. On September 3 a female was examined in which the eggs, surrounded by their gelatinous envelopes, had all collected in the lower part of the oviduct — the "uterus" — which was much distended, spindle-shaped, about 10 cm. long by 4 cm. in diameter at its widest part; its thin walls had a rich blood supply.

The preparations of the males for the breeding season proceeded at equal pace with those of the females. On August 31, in a specimen 38 cm. long, having the glandular region about the cloacal opening very much swollen, the vas deferens was found to be much distended with milt, and measured about 8 mm. in diameter. From this specimen the seminal fluid could be easily stripped. The spermatozoa were motile; the motion of the shaft is slow as compared with other forms, but that of the undulating membrane is rapid. The posterior third of the testis was darkened and shrunken, and appeared much degenerated; as stated by McGregor ('99), a wave of maturation begins at the posterior end of the testis and passes forward.

Hence the breeding season in this locality may be said to begin about the last of August. Possibly it is influenced by temperature; cool nights, accompanied by a marked decrease in the temperature of the water, set in three or four days previously. During the breeding season the temperature of the water ranged from 14° to 18° C. The egg-laying season lasts about two weeks.

Oviposition. — In a ripe female, the string of eggs in the uterus is aggregated in a much twisted and tangled mass. The egg capsules at the end of the uterus nearest the cloaca, hence those first formed, contain no eggs; likewise those nearest the oviduct proper, hence the last formed, are empty. The egg envelopes at this time are very soft, much wrinkled, contain no water and therefore fit closely about and between the eggs, taking up very little extra space. The number of eggs in a single uterus was counted in one specimen of average size and found to number 220; in another specimen of equal size 225. Those remaining in the ovary were very small (about 1–2 mm. in diameter), hence could not mature until another season. Therefore the number of eggs deposited by a female of average size in a single season must be about 450 — probably more in the case of a large female.

The strings of eggs after deposition are usually found twisted together in a tangled mass, corresponding to their condition in the uterus. The process of egg-laying was observed in aquaria in several instances. Egg-laying generally begins slowly, a short string of capsules containing from six to a dozen eggs protruding from the cloaca for many hours before the main mass is deposited; the majority of the eggs are deposited more rapidly, in a constant stream, the process requiring only about five minutes. The newly laid egg capsules take up water slowly, so that for a day or two the envelopes remain flaccid and much wrinkled. They gradually become plump and turgid (see Fig. 5) by osmosis, affording much more efficient protection to the eggs; the folding of the envelope almost entirely disappears. When the eggs are removed from the water, the egg proper looks much larger than it really is, because magnified by the spherical capsule at the bottom of which it lies.

As already recorded (Smith, '06[2]), of the two spawnings of eggs found during the autumn of 1905, one lot of eggs was in the open, the other partly under a rock. The eggs were not searched for under rocks at this time. In the season of 1906, about a dozen nests were found, all in cavities under rocks, and only a few scattering eggs were found in the open. Hence the deposition of eggs under rocks is the normal procedure, and their occurrence in the open quite exceptional. The "nest" of *Cryptobranchus* is simply the ordinary dwelling-place of the animal, used for spawning purposes.

The absence of black pigment in the eggs is probably correlated with the fact that they are laid in darkness. The eggs of *Necturus*, which are also laid under cover, are likewise devoid of pigment.

During the breeding season the water is unusually low. In one case a nest was found at the extreme water's edge, so that the eggs were barely immersed, yet most of them were developing. In this way, by the periodical drying up of the water in swamps, may have originated the terrestrial nesting habits of *Amphiuma*, described by Hay ('88).

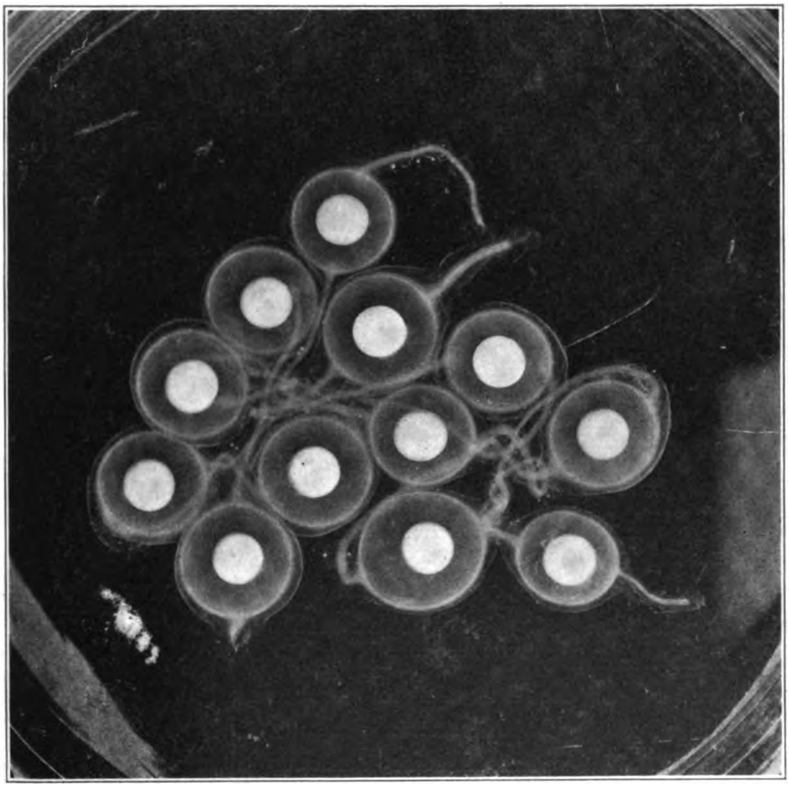


FIG. 5. Unfertilized eggs of *Cryptobranchus alleghenienisis*, after two days' immersion in water. Natural size.

Fertilization. — In a previous paper ('06[2]), the conclusion was reached that fertilization in *Cryptobranchus* is internal. The evidence upon which this conclusion was based was afforded by the usual experiment of isolating a ripe female; an hour afterwards a few fertilized eggs were found, although their deposition was not directly observed. It was not known at the time that the female, as well as the male, devours the eggs of its own kind; moreover, the possibility of the regurgitation of the eggs, and their subsequent development, did not occur to me; yet this is undoubtedly what happened. The female *Cryptobranchus* is a voracious eater of the eggs of her own kind; digestion is very slow, and if the animal soon after eating fertilized eggs, is placed in quiet water, the eggs will be regurgitated without injury, and have been observed to continue their development. The number of eggs found on September 6, 1905, was rather large (estimated at about 80) to have been contained in the stomach at one time; but the female was an unusually large specimen, and the eggs must have been devoured before the inflation of their envelopes; hence the feat was quite possible. The evidence for internal fertilization cited above must be regarded as of no value.

The study of the breeding habits was begun with the expectation of finding spermatophores, such as are known to be deposited by *Triton viridescens* (Jordan, '91 and '93) and many other Urodeles (Wiedersheim, '00). Had such spermatophores existed it is quite probable that I should have recognized them through familiarity with spermatophores of *Triton viridescens*, deposited in aquaria, and observations (Smith, '07) on the spermatophores of *Amblystoma* studied in the field; yet, although strings of mucus containing spermatozoa were found floating in the water attached to rocks in localities where *Cryptobranchus* abounds, no spermatophores were found. The search for them was persistent and thorough, both under rocks and in the open, and in creek aquaria where the animals were breeding in large numbers; yet always with the same negative results.

Laboratory studies were conducted .to shed light upon the process of fertilization. Eggs taken from ripe females uniformly failed to develop; no spermatozoa were found, either in the cloaca of the female or in eggs taken from the uterus. Females in the act of laying eggs were seized and some of the remaining eggs stripped from the cloaca; no spermatozoa could be found in them and they did not develop. The "opaque body" previously mentioned (Smith, '06[2]), which can be dimly seen in the photograph (Fig. 5), was found to have no essential significance in the process of fertilization, since it is entirely a product of the female, and uniformly present in unfertilized eggs. As stated by McGregor ('99), the female has no seminal receptacle.

Tests were made in order to determine how long living spermatozoa would survive exposure to water. In seminal fluid taken from the vas deferens and thoroughly mixed with water, motion of the sperms, both of the shaft and the filament, continued for about 15 minutes. In drops of seminal fluid obtained by stripping and consequently mixed with the viscid secretion of the cloacal glands, the motion of the sperms continued for four hours after immersion in water.

Artificial fertilization was attempted on several occasions, always with complete success. Eggs for this purpose were secured by killing and cutting open ripe females. Three methods were followed: (*a*) "dry" fertilization — mixing eggs and milt together thoroughly, then immersing in water; (*b*) eggs and milt were placed simultaneously in water, then mixed together; (*c*) eggs were immersed in water for a minute or two, then milt added. All three methods were successful. In every case eggs not artificially fertilized were kept as a check; in no case did these develop. Hence it was proved that the sperms are able to penetrate the egg capsule from without, and that fertilization may take place after both eggs and sperms have been exposed to water.

All the evidence pointed to external fertilization, and it was sought to verify this by actual observation.

With the beginning of the breeding season a marked change took place in the behavior of the animals in their natural environment; they no longer remained secluded in hiding-places under rocks, but came out boldly by day, sometimes congregating in droves of from six to twelve, showing a social instinct quite lacking during the summer. As a rule they roamed restlessly about, poking their noses into crevices under rocks, as if exploring; sometimes, however, they lay quiet in the open. Several times they were observed to pile up in crevices between rocks, two or three lying alongside each other, or two or more at a time trying to force their way into the same crevice; this, however, may have been merely incidental to the favorable location. Those confined in aquaria were less shy than formerly, and paid little attention to the presence of an observer; this was true of newly captured specimens as well as of those that had been kept for some time.

Finally, in a large creek aquarium, under conditions made as natural as possible without affording too much cover, the complete process of fertilization was observed at close range in two instances, while various details of the behavior of the animals during spawning were repeatedly observed. The process is essentially as follows:

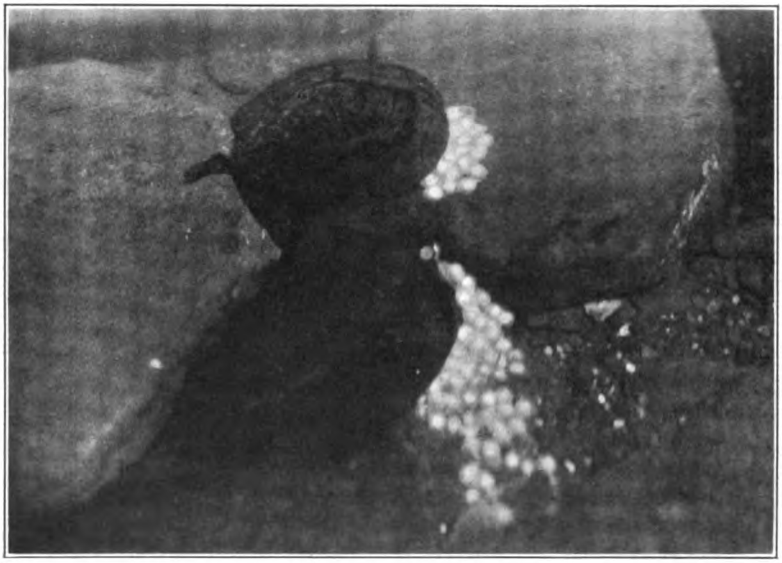


FIG. 6*A*. Photograph from under-exposed negative, showing spawningof *Cryptobranchus allegheniensis*. For explanation see Fig. 6*B*. The photograph was taken with the aid of a water-glass.

The female, with a short string of eggs protruding from her cloaca, crawls restlessly about, dragging the eggs along the bottom; the male, whose attention seems to be attracted by the bright yellow eggs, follows her. Sometimes the female stops, and makes swaying lateral and vertical movements with the posterior part of the body. Finally she crawls partly (in the cases observed) under a rock, and egg-laying begins in earnest. During the remainder of the process of spawning the female lies quite motionless. The male moves to a position alongside and sometimes slightly above the female and overlying the eggs, or sometimes beside them. In this position, he makes swaying lateral and vertical movements of the posterior end of the body, raising and lowering it with his hind legs, much like a male *Triton viridescens* depositing a spermatophore, except that in the case of *Cryptobranchus* the motion is less violent. The movements also resemble those of the female preliminary to spawning. While executing these movements the male extrudes from his cloaca a snowy-white ropy or cloudy mass which consists of seminal fluid mixed with the secretion of the cloacal glands. On one occasion the deposit occurred in ropy chunks about 4 mm. in diameter and 2 or 3 cm. long. The deposit does not necessarily fall directly on the eggs, but sometimes on the ground beside them. Soon after, his own movements or those of other male hellbenders which approach brush the material about until

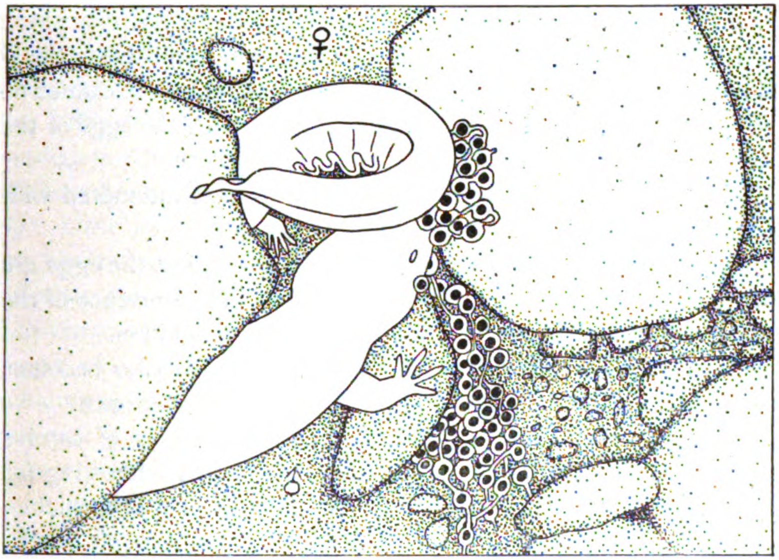


FIG. 6*B*. Drawing explanatory of Fig. 6*A*. The female, in a curved position has her head under a rock; the male, advancing from under the rock, with his foot on a stone, is not yet in position to fertilize the eggs.

it becomes diffused amongst the eggs. It usually happens that while a female is spawning several males are lurking about taking an evident interest in the proceeding. Once, while one male lay amongst the eggs (whether he fertilized them could not be seen) another male about a foot away, and certainly not in contact with the female, was observed to deposit milt. Evidently the excessive number of males is of value to insure fertilization. After fertilizing the eggs, the male sometimes leaves them, but more often remains beside them, or crawls under or amongst them.

Under perfectly natural conditions, spawning and the fertilization of the eggs takes place under large rocks where it cannot be observed; also the male more often remains with the eggs in the normal nesting place.

Photographs of the spawning operations were attempted with poor success (see Fig. 6).

Fertilization is then external as in most fishes, but the eggs are deposited without direct aid from the male. The presence of the string of eggs seems to be sufficient for sex recognition.

With the close of the breeding season, *Cryptobranchus* becomes more shy, avoids the light, and is seldom seen in the open.

Kerbert's account ('04) of the behavior of a pair of captive specimens of *C. japonicus* during spawning will be interesting for comparison. "Schon zu anfang des August 1902 verhielten sich die beiden Tiere anders als gewöhnlich. Wahrend die durchaus trägen, stumpfsinnigen Geschöpfe in der Regel tage- und wochenlang bewegungslos, fast wie tot, auf dem Boden ihres Behälters lagen, nur äusserst langsam nach den ihnen dargebotenen Fischen schnappten, das Licht scheuten und immer die dunkelsten Stellen ihres Behälters aufsuchten, fingen dieselben im August an sich einander zu nähern und gegenseitig zu berühren. Manchmal wurden zitternde und wellenförmige Bewegungen des ganzen Körpers wahrgenommen.

Die Vermutung lag auf der Hand, dass ein Erregungszustand des Nervensystems als Einleitung zur Zeugung eingetreten war. Das Liebesspiel dauerte nur einige Tage. Eine eigentliche Begattung habe ich nicht beobachtet — und auch nicht erwartet." (Kerbert naturally expects fertilization to be accomplished by means of spermatophores). . . .

"Nachdem das grössere, oder — wie ich nacher festzustellen in der Lage war — männliche Tier (1 m. lang) schon seit Anfang September eine unverkennbare Unruhe gezeigt und im Sande am Boden seines Behälters eine deutliche Grube oder Vertiefung gewühlt hatte, fing am 19 September — es war ungefähr sechs Uhr Nachmittags — bei dem kleineren Tiere die Ablage der eigentümlichen Eiermasse an. . . .

Wahrend der Eiablage schwamm das Weibchen in merkbarer Unruhe herum, legte sich aber nach Beendigung dieses Vorganges ganz ruhig hinter den Felsen an der Hinterwand des Behälters. Das grössere Tier war vom Anfang an weit unruhiger und mehr aufgeregt als das Weibchen, schwamm fortwährend durch die von den heftigen Schwimmbewegungen beider Tiere allmählich in die sandige Grube geratene Eiermasse und wehrte die kleinen Fische, Mitbewohner des Behälters, mit geöffnetem Maule von den Eiern ab. Obwohl er sich einige Minuten später scheinbar ruhig bei der Eiermasse hinlegte, war die Erregung des Nervensystems doch offenbar eine so intensive, dass die Haut des Rumpfes und des Schwanzes wellenförmige, zitternde Bewegungen zeigte, ja dass sogar eine heftige Ejaculation von Sperma erfolgte. Eine schleimige grauweisse Masse machte das Wasser trübe. Bei mikroskopischer Untersuchung stellte sich unverkennbar heraus, dass in dieser schleimigen Masse eine grosse Anzahl von Samenfäden anwesend war. Mit voller Bestimmtheit war also nachgewiesen, dass das grössere Tier wirklich ein Männchen war." No deposit of material in a definite mass to form a spermatophore was observed. Kerbert rejects the idea that the emission of seminal fluid on this occasion indicated external fertilization of the eggs laid at that time, and suggests that the spermatozoa diffused in the water might be taken up into the female cloaca to fertilize the eggs for another season; or that the extrusion of milt at this time has no significance in fertilization, being merely incidental to the excitement of the male on account of the spawning of the female. Ishikawa ('04) also inclines to the belief that fertilization is internal, though without conclusive evidence. In view of the results obtained with *C. alleghenienis* it seems very probable that the observations cited above indicate external fertilization in *C. japonicus.*

Considering the low taxonomic position of *Cryptobranchus* amongst the Urodeles, the occurrence of external fertilization in this form indicates that this is the primitive method for the group; internal fertilization by means of spermatophores, characteristic of so many Urodeles, is a later development.

Nothing conclusive is known concerning the method of fertilization in the Crossopterygian Ganoids, the ancestral stock from which the amphibia are believed to be descended. In the higher Teleostomi, with a few notable exceptions, fertilization is external. In the Dipneusti, which, like the Amphibia, are supposed to be derived from a primitive Crossopterygian form, nothing definite is known concerning the manner of fertilization. In view of the diversity of habits within such groups as the Teleostomi and the Urodela, and the wide gaps between the Urodeles and the nearest living representatives of ancestral groups, it is probable that even were our knowledge of the habits of existing animals complete, a comparison between the Urodeles, the Crossopterygii, and the Dipneusti would have little phylogenetic significance. On the other hand, the occurrence of a simple method of external fertilization as the primitive condition of the Urodeles suggests the possibility of finding within the group stages in the development of the complicated breeding habits, involving internal fertilization by means of spermatophores, characteristic of most of its representatives.

Brooding Habits. — It has been noted previously that the male usually remains with the eggs after fertilizing them. That the male may render efficient protection to the newly-laid eggs is shown by the following incident of September 1, which I quote almost verbatim from my notes.

A large "red" hellbender (afterwards identified as a female) crawled across the stream toward a flat rock submerged in about 10 inches of water. This rock was about 3 feet by 2½ feet, slightly tilted so that the down stream side was a little raised from the bottom. As the female approached this rock, the large flat head of another *Cryptobranchus* (afterwards found to be a male) of unusual size was thrust out from under the down-stream edge of the rock and the newcomer was seized at the side of the head by the jaws of the male. She drew back and was released.

She went away a short distance but repeatedly returned and was repulsed in the same manner. Another small specimen (afterwards found to be a male) joined in the attack, but was also seized and repulsed; once he effected an entrance, whereupon a struggle ensued beneath the rock, as was shown by the water becoming turbid. The intruder withdrew.

The large female returned to the attack. She was seized with a firm grip, which lasted several minutes, during which the combatants rolled over and over, and sometimes drifted with the ventral surfaces uppermost. When released, the female swam away for a rod or more, and did not return.

The large male returned to the rock, but kept his head exposed, as if watching for another attack. Sometimes he held his head erect in an aggressive attitude like an angry snake, although it is possible that this was only a movement preliminary to rising for air. At my approach, he assumed this apparently threatening attitude, contrary to the usual custom in such cases of remaining motionless, or withdrawing under the rock.

All the specimens concerned were captured. The rock was overturned, and beneath it was found a cavity containing a large mass of eggs which were immediately swept down stream by the current. When collected they were found to number nearly 600, in first and second cleavage stages.

Another small male and a small *Necturus* were also found under the rock, but not in the cavity containing the eggs; they were on the side most remote from that occupied by the large male. Presumably their presence had escaped his attention, occupied as he was with his enemies.

The large male had a greatly swollen cloacal region, and his struggles when handled caused milt to exude from the cloacal opening. The stomachs of all the specimens excepting the small male found under the rock (the *Necturus* was not examined) were found to contain eggs; the large male contained 23 eggs, the female 22, and the small male 10. In the case of the female, strings of eggs protruded from the mouth and from the branchial aperture. Since the female was found by examination to be a spent one, and spent females at this date were rare, it is very probable that the eggs were laid by her. Even while I was gathering the scattered eggs, another hellbender appeared on the scene and attempted to devour them.

As previously stated, the eggs were in the first and second cleavage stages, and appeared to represent a single spawning. This was the first nest found during the season, and no other nest was found near it.

In the majority of nests found containing eggs in an early stage of development a male was present, a female never. In one case a male was found in a nest containing embryos two or three weeks old; but there is no certainty that he had remained there continuously during that time, or that the embryos were his own offspring.

The number of eggs found in the stomach of a single *Cryptobranchus* usually ranges from 15 to 25; in a few cases this number was considerably exceeded. The digestive processes of the hellbender are extremely slow, and I have taken undigested eggs from the stomach a week after they were eaten.

So far as observed, only recently laid eggs were eaten by the adults. After eggs have been laid several days they are rendered inconspicuous by a covering of silt.

As previously stated, the number of eggs usually deposited in a single nest is about 450 to 500. Nests found late in the season, with eggs in an advanced stage of development, contained nearly the full number of eggs; probably not more than a tenth part, in most cases, had been eaten.

We have here the beginning of a paternal brooding habit, but only the beginning. A male in making a valiant defense of the nest protects the eggs, to be sure, but at the same time he guards his own food supply. Thus in the case of *Cryptobranchus* the brooding habit may have its origin in the feeding habit. On account of the slowness of his digestive processes, and the short period during which, it appears, the eggs are available as food, the male hellbender alone is unable to eat more than a small portion of the eggs. The habit of defending the eggs during the early stages of their development is presumably, even though some of them are eaten, of advantage to the species; and this guarding habit may be initiated in connection with the feeding instinct.

In objection to this interpretation it might be urged that an animal that had just eaten would not be likely to quarrel over more food; but as a matter of fact the hellbender caught attempting to eat the scattered eggs was already gorged with them.

Another possible interpretation demands consideration. In the case described, the male may have been merely holding the nest while awaiting the coming of another ripe female. The hellbenders driven away were all males with the exception of a spent female; there was no opportunity to observe the reception of a ripe female by a male guarding eggs. Evidently further observations are needed to supply conclusive evidence of the origin of the brooding habit.

The aggressiveness displayed by the male *Cryptobranchus* in defending the nest is the first instance I have observed of pugnacity on the part of a Urodele. In these contests over the possession of the nest, the male doubtless has an advantage over a spent female, since the latter seems to be in a weak condition immediately after spawning.

Concerning the brooding habits of *C. japonicus*, Kerbert ('04) says: "Nach Beendigung der Eiablage legte sich das Weibchen offenbar in grösster Ermattung in eine Ecke des Behälters hin und kümmerte sich um das Gelege gar nicht mehr. Das Männchen hingegen hat seitdem die Eiermasse nicht verlassen — ja sogar *die Brut fortwährend bewacht*. . . . Denn sobald das Weibchen der Eiermasse zu nahe kam, stürzte das Mannchen in sichtbarer Wut auf die Mutter los und vertrieb sie . . . kriecht der Männliche Riesensalamander zwischen den verschiedenen Strängen der Eiermasse hindurch und bleibt dan von der Eiermasse umhüllt liegen, oder er legt sich einfach neben die Eiermasse hin. In beiden Fällen aber hält er, hauptsächlich durch eine pendelartige Bewegung des ganzen Körpers, von Zeit zu Zeit die ganze Eiermasse in Bewegung. Durch diese Bewegung entsteht eine für den Atmungsprozess der Eier und Embryonen höchst wichtige Wasserströmung, während die Lage der Eiermasse hierdurch gleichzeitig fortwährend wechselt."

III. The Embryo.

An abundance of fertilized eggs was collected, but on account of the lack of proper facilities great difficulty was experienced in keeping the material alive long enough to secure a complete series. By far the greater part of the eggs perished from variations in temperature, insufficient aeration, or the attacks of water-mold, and it was only by constant search for new nests that the later stages were obtained.

Segmentation.— The segmentation stages have already been described (Smith, '06[2]), and since I expect to make a detailed study of the embryology the subject of a special paper, only a few brief notes on the development of the embryo need be given here.

In artificially fertilized eggs the first cleavage furrow appears about 18 hours after fertilization. Segmentation proceeds with increasing rapidity, varying with the temperature, and in a few days a blastula is formed having a shallow segmentation cavity with a very thin, almost transparent roof. So far as external changes visible to the unaided eye are concerned, development during the latter part of this process is comparatively slow. It is also a critical period in the development of the egg ; more embryos die at this time if exposed to slightly unfavorable conditions than at any other.

Gastrulation commences in from six to ten days, according to temperature, after fertilization. The blastopore is formed much as in the frog's egg. The process is quite rapid after the appearance of the short deep horizontal groove just below the equator; this groove quickly elongates, becomes crescent shaped, and the horns close to form a complete circle in some eggs, while in others of the same lot subjected to the same conditions, the process of gastrulation has just begun. The yolk plug rapidly diminishes in size.

Origin of the Nervous System.—The broad but shallow neural groove appears at about the time the circle of the beginning blastopore is nearly complete—a day or two after gastrulation commences. A few hours later the neural folds appear as a sharply-defined horse-shoe shaped ridge about the upper end of the neural groove ; the arms extend downward and closer together until they reach the dorsal lip of the blastopore, which by this time has narrowed to a very small circle. The development of the neural folds proceeds rapidly until their closure a day or two after their first appearance.

Eighteenth Day Embryos. — In from two to three weeks after fertilization the embryos reach the condition represented in Fig. 7. At about this time the rudiments of the external gills appear. A broad horizontal pink band along the left side of the yolk sac indicates the beginning of the vitelline vein. The dorsal surface becomes slightly pigmented, the pigment spots being grouped metamerically (cf. McGregor, '97). The embryos squirm slightly when placed in the fixing fluid.

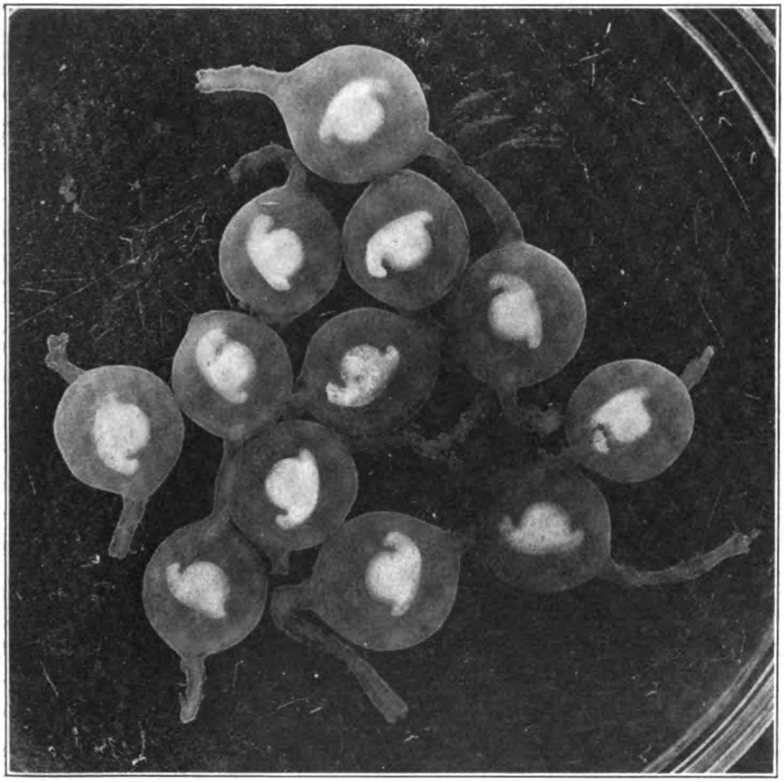


FIG. 7. Embryos of *Cryptobranchus alleghenienis*, about 18 days old, within egg capsules. . Natural size.

Observations in the field were necessarily discontinued at this stage, but living material was transported to the Zoölogical Laboratory of the University of Michigan. The temperature during transportation was regulated with ice. After their arrival at the laboratory the embryos were kept in filtered water aerated with an aspirator, in dishes partly immersed in cool running water. The loss both during and after transportation was very slight.

Four Weeks' Embryos. — Embryos a month old have attained a length of about 20 mm. The eyes are prominent and pigmented; the entire dorsal side of the body is well covered with black pigment, though the pigment is far from being as dense as in an *Amblystoma* or a frog at a corresponding stage of development. Lateral line sense organs can be distinguished. The three pairs of external gills are well fringed and pink with blood; the blood corpuscles are large and may be easily observed with a hand lens. The vitelline vein, now very prominent, has shifted to a position along the lower part of the left side of the yolk sac. A conspicuous red spot marks the position of the heart.

Spontaneous movements (exercise movements) now occur. These consist of jerking the head from side to side; wriggling; reversal of the laterally curved position of the body by turning over; swimming movements in which the embryo butts against the envelope and subsides; swimming in a circle. Embryos removed from the capsule at this stage make practically the same movements; they are unable to progress in a straight line and incapable of prolonged swimming movements.

Hatching. — Embryos collected when about three weeks old and kept in the laboratory at a temperature averaging about 13° C., began to hatch on October 17, about six weeks after the eggs were probably laid. Nearly all were hatched by October 24. At this time the average length was about 25 mm. The appearance of the embryos just previous to the hatching is shown by Fig. 8.

Before the hatching period, the envelopes become much softened and considerably enlarged by the absorption of water, making room for the growing embryo. The latter sometimes escapes by pushing its way through the envelope, leaving a small round hole; sometimes apparently by tearing or bursting the envelope by means of its wriggling movements.

In its natural environment the hatching of the embryo is perhaps delayed a little by the slightly lower temperature, but the climatic conditions are such that it seems at least certain that hatching occurs at some time during the fall.

According to Kerbert ('04), the eggs of *C. japonicus*, kept at an average temperature of 13° C., hatched in from 52 to 68 days, and at the time of hatching the embryos were 30 mm. long. Since the extreme length of the adult *C. japonicus* is 159 cm. (Gadow, '01) as compared with 56 cm. for *C. alleghcniensis*, there is far from being a corresponding difference in the size of the embryos at the time of hatching.

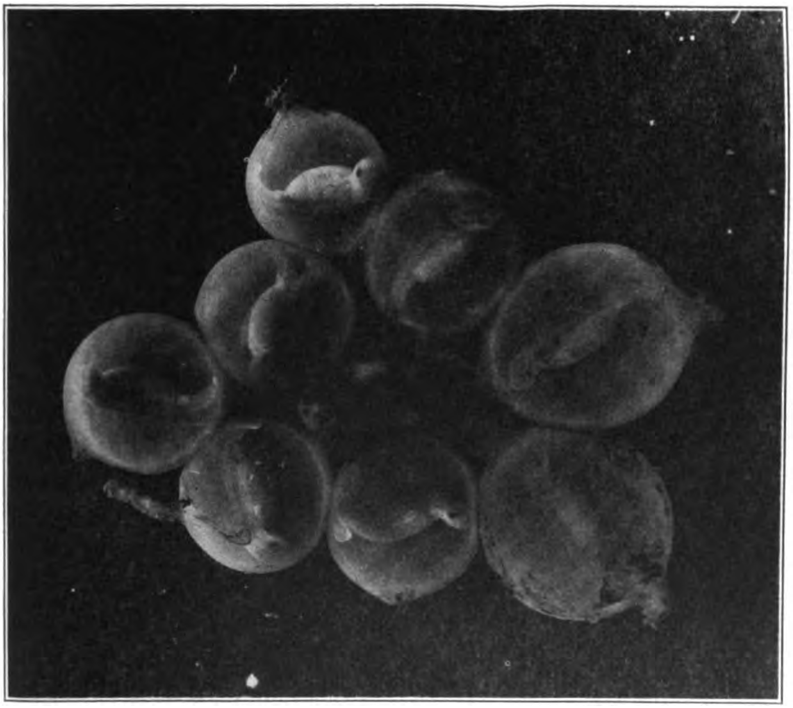


FIG. 8. Embryos of *Cryptobranchus allegheniensis*, ready to hatch, about six weeks old. Natural size.

IV. The Larva.

A. *Early Stages Studied in the Laboratory.*

The Newly Hatched Larva. — At the time of hatching the larva (see Fig. 9) retains a large yolk sac. The dorsal surface is well pigmented, but the larva is pale as compared with amphibians that live exposed to the light. The ventral surface is lacking in pigment, leaving the abdominal region yellow from the presence of yolk and the throat region white and nearly transparent. The heart can be readily observed without dissection. The vitelline

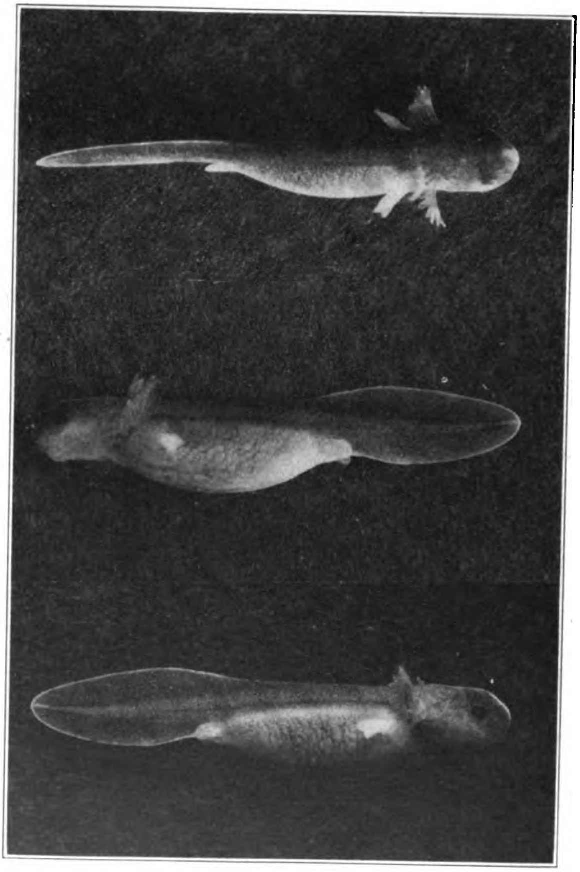


Fig. 9. Newly hatched larva of *Cryptobranchus alleghaniensis*, 25 mm. long, photographed while living. × 2½.

vein is very prominent. The anterior limb rudiment is provided with two toes. The somites are plainly visible in most specimens, but do not show in the figure. The mouth opening is large and ventrally situated, and the mouth cavity is well developed. The eyes are more prominent than in the adult. The dorsal and lateral surfaces, especially of the head, are thickly studded with small round white spots, presumably sense organs and mucus glands.

On account of the large yolk sac, the larva habitually lies on its side, turning occasionally from one side to the other. When the larva is placed upon its back, the righting reaction, aided as it is by the ballast of yolk, is very quick. The newly hatched larva is able to swim rapidly in a straight line for a short distance, using the tail as a propeller.

The larvæ avoid the light and are positively rheotactic. Under natural conditions, the result of these two modes of behavior probably is that the larvæ remain in the nest.

Aeration of the blood is afforded, not only by the external gills, but by the capillaries which lie close to the surface all over the body. The tail may be of especial importance in respiration, for here, as in the external gills, the capillary network lies in close proximity to the water on both sides.

At the time of hatching, patches of cilia are scattered over practically the entire surface of the body, but are especially numerous on the gills. Their beat is backward, and they create a current of water, subservient to respiration, which is particularly strong in the vicinity of the gills. The presence of cilia on the ectoderm of Amphibian embryos has been remarked by various writers (see Assheton, '96).

The Month-old Larva. — In larvæ about a month after hatching (see Fig. 10), there was noted a marked increase in length, principally due to the development of the tail. The larvæ now measure from 30 to 35 mm. There is also a rapid elongation and development of the front limbs, which now have four toes. The form and position of the front limbs adapt them for use as paddles. The posterior limbs develop more slowly, are relatively short and have but three toes distinctly visible, though in some cases rudiments of the fourth and even the fifth are present. The yolk sac is reduced enough so that the larva no longer lies on one side. The mouth opening is now only slightly ventral. Pigmentation is greatly advanced, and extends to the external gills; when viewed from above the larva is now nearly black. The ventral side remains white and nearly transparent in the throat region and yellow in the region of the abdomen or yolk sac. The heart is still visible, and the course of the blood through it may be readily followed. The vitelline vein, now lying almost in the median line ventrally, shows signs of degeneration.

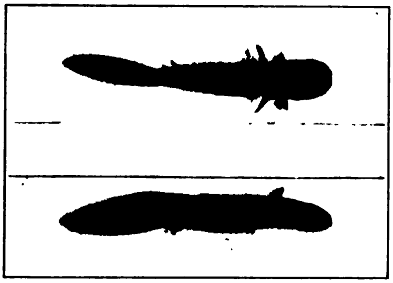


FIG. 10.

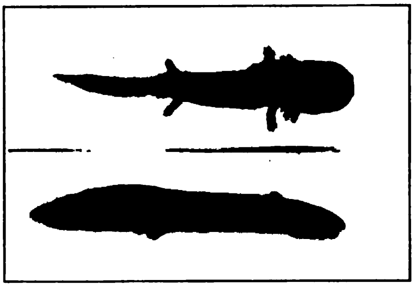


FIG. 11.

FIGS. 10 and 11. Photographs from living material, showing early stages in the development of the larvæ of *Cryptobranchus allegheniensis*. Natural size.

FIG. 10. Larvæ one month after hatching. Length 35 mm.

FIG. 11. Larva ten weeks after hatching. Length 40 mm.

At this age the larvæ are more active swimmers than the adults. The front limbs assist in starting by a quick simultaneous backward stroke.

Ciliation of the epidermis is now found only on the gills, where a strong eddying current of water is produced. No water current in at the mouth or nares and out through the gill slits could be detected.

At this stage shedding of the cuticle was observed for the first time, and was quite general; the water of the aquaria became cloudy with portions of detached epidermis.

Ten Weeks' Larva. — (See Fig. 11.) There is a slight increase in length since the first month; the larvæ now range from 35–40 mm. The limbs are better developed, possess the full number of toes, and are used in walking in the same manner as in the adults. The limbs are broad and flat, and are used as paddles in swimming at a moderate rate of speed. After fixation the somites show very distinctly.

The yolk sac is greatly reduced, and in some specimens the abdomen presents a shrunken appearance. The vitelline vein is no longer visible. The larvæ seize small pieces of beef offered to them, but do not swallow them.

Six Months' Larva. — Larvæ six months after hatching measure about 40 mm. From the third to the sixth month the increase in length is very slight, but the larva becomes much stouter in structure.

After the fifth month the larvæ not only seize small pieces of raw beef moved along a little to one side of the head, but, to a limited extent, swallow and retain these morsels. The presence of feces in the water indicates that some of the meat is digested.

In larvæ of this age fixed in Tellyesnicky's fluid and preserved in alcohol, the lateral line organs are quite conspicuous; their distribution can readily be made out with the naked eye.

Neither the larvæ of this nor of earlier stages have been observed to come to the surface for air.

The course of larval development described above may be somewhat slower under natural conditions on account of lower temperature. During the period under consideration the temperature of the aquaria ranged from 13° to 9° C., while that of the stream from which the specimens came ranged from 10° to 5° C. It is very probable that the larvæ hibernate in the nest, and perhaps do not emerge until late in the following spring. I have found clusters of 35 mm. *Necturus* larvæ late in August, occupying what was apparently their original nest.

At the time of writing a considerable number of larvæ are being kept alive in the laboratory, and it is hoped that some of them may be reared to the adult condition.

B. *Later Stages Studied in the Field.*

During August of the past summer search was made for larvæ in their natural environment. A few larvæ were found beneath small flat stones in running water only three or four inches deep.

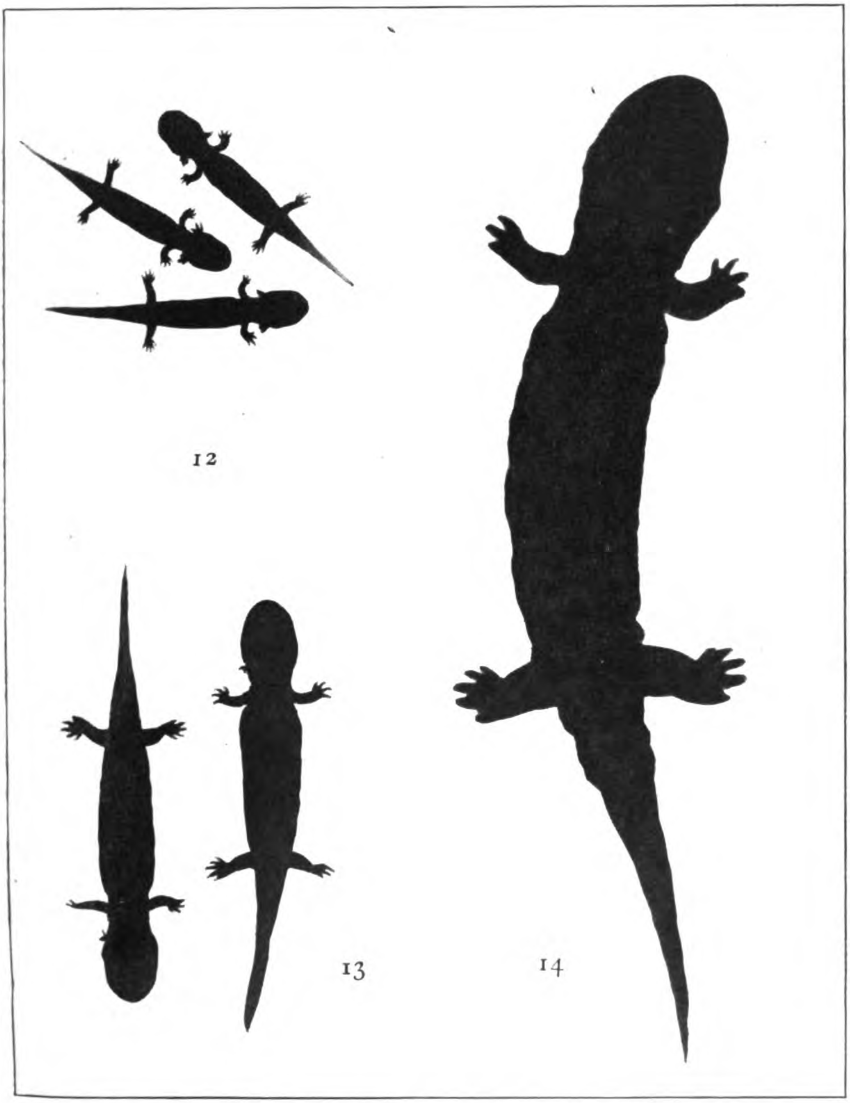
The Year-old Larva.— The smallest specimens obtained (see Fig. 12) were about 7 cm. long. Four specimens were found measuring as follows; 6.4 cm., 6.8 cm., 7.0 cm., 7.3 cm. These were presumably hatched during the preceding autumn. The external gills are retained, though evidently in a somewhat degenerate condition. The legs are well developed. The mouth opening is terminal, not ventral. The tail is proportionally smaller than in specimens ten weeks old; the ratio of length of tail to entire length is 1 : 2.5 in the case of the year-old larva, and 1 : 3 in the ten-weeks' larva. The body is plump as compared with the adult, the somites show distinctly, and the lateral folds are very slightly developed. There is a conspicuous gular fold not present in the adult. The color of the dorsal and lateral surfaces is a very dark brown, almost black, so that the irregular black spots which are present do not show in the photograph. After preservation in formalin the ground color becomes lighter and of a bluish tint, and the black spots show more distinctly. A few scattering inconspicuous yellow spots are also present. The ventral surface is considerably lighter in color.

These larvæ are more active than the adults, and are extremely sensitive to shocks and jars. They were never observed to come to the surface for air.

The Two-year-old Larva.—(See Fig. 13.) Two specimens were found, measuring respectively 12 cm. and 12.3 cm. These differ from the year-old larvæ in the small size of the external gills, in the absence of the gular folds, the slightly greater development of the lateral folds, and in the absence of visible somites, though the lateral folds are metamerically notched. The ground color is a trifle lighter, so that the black spots are more clearly seen; less conspicuous yellow spots are also present. No stages intermediate between these and the 7 cm. larvæ were obtained, and it seems probable that they represent the second year of larval development.

One of these specimens ate, soon after being captured, a large *Corydalis* larva. Another specimen, when placed in quiet water, regurgitated a partly digested 6 cm. larva of its own kind.

As in the younger stages, these specimens were not observed to come to the surface for air; however, the evidence on this point is not conclusive.



FIGS. 12–14. Larval and post-larval stages of *Cryptobranchus alleghenieusis*, ½ linear reduction. From living specimens.

FIG. 12. Larvæ taken August 14, photographed August 17, 1906. Length 6.4 cm., 6.8 cm., and 7.0 cm. respectively.

FIG. 13. Larvæ taken August 14, photographed August 17, 1906. Lengths 12.0 cm. and 12.3 cm. respectively. Reduced external gills are present on both sides.

FIG. 14. Young *Cryptobranchus* 26.7 cm. long.

V POST-LARVAL DEVELOPMENT.

A series of sizes intermediate between the 12 cm. larvæ and the adults was obtained. Six specimens taken during a single week in August measured as follows: 14 cm., 16.2 cm., 18.3 cm., 21 cm., 21.5 cm., 26.7 cm. All these had lost their external gills, but were sexually immature. The lateral folds show increased development throughout the series. The coloration is about the same as that of the young adult; Fig. 14 will serve to show the general appearance of the specimens ranging from 16 to 35 cm., in which the spots are more conspicuous than in either very young or very old specimens.

Sexual maturity is attained with a length of about 34 cm., and probably requires three or four years from the time of the fertilization of the egg.

In conclusion, I take pleasure in acknowledging my indebtedness to Professor Jacob Reighard for training in field work without which it would have been impossible for me to conduct studies of this kind far from the advantages of a laboratory, and to Dr. O. C. Glaser, under whose direction work was continued at the university, for invaluable encouragement and advice.

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THE SPAWNING HABITS OF CHROSOMUS ERYTHROGASTER RAFINESQUE.*

BERTRAM G. SMITH.

While a student at the University of Michigan during the years 1904–1907 I acted as assistant in zoölogy and when accompanying Professor Jacob Reighard on field excursions had many opportunities to observe, under his guidance, the breeding habits of minnows and other fishes. This familiarity with the unpublished work of Professor Reighard made it possible for me to make the observations recorded below, which are published with his permission.

About the middle of May, 1907, I found the minnow *Chrosomus erythrogaster* Rafinesque very abundant in a small brook near Lake Forest, Ill. As the males were in breeding dress I seized the opportunity to make some observations on the spawning habits.

Habitat. — The brook flows through a large open pasture and also through woodland; the minnows were far more numerous in that portion of the brook which is in the open field. Here the stream meanders through a shallow valley, over a pebbly or sandy bottom. In places it is so narrow that one can readily step across it; elsewhere it expands into pools or shallow rapids not more than six feet wide. In the rapids the water is seldom more than two or three inches deep; in the pools it may reach a depth of one or two feet. The current is quite rapid; even in the pools there is little quiet water.

Chrosomus is by far the most abundant fish in this brook. Schools of from a dozen to several hundreds abound, while of other fishes only a few large suckers, and an occasional *Rhinichthys*, *Semotilus* and *Cottus*, were observed.

Sexual Dimorphism. — The bright colors of the males were scarcely noticeable when the fishes were in the water and viewed obliquely from above; but when removed from the water the males were found to be marked on each side of the abdomen with a broad longitudinal stripe of the most vivid and brilliant scarlet that I have ever seen. This stripe starts just back of the operculum and runs immediately below the lower of the two lateral dark bands (see Fig. 1) and parallel to it, reaching almost to the caudal fin. There is also a small red spot just below the mandible on each side. In some specimens the lateral bands of red are faint except just behind the operculum. In the more highly colored males the entire abdomen is covered with red, and there is a red spot in the anterior part of the root of the dorsal fin. The pectoral and pelvic fins, and the anal fin, are bright lemon yellow; the dorsal fin and tail are faintly marked with pale yellow. There is a small spot of yellow on the ventral side of the body at the base of the caudal fin, and another in the gular region.

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* Contributions from the Zoölogical Laboratory of Syracuse University.

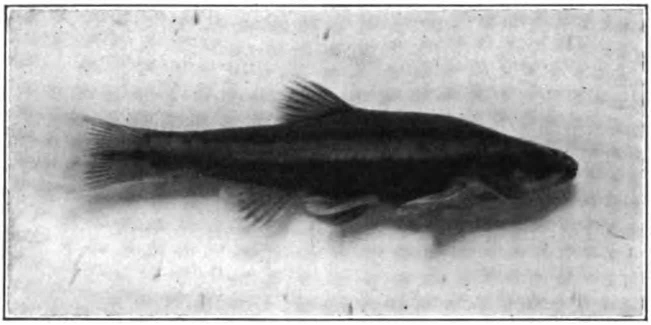

FIG. 1.

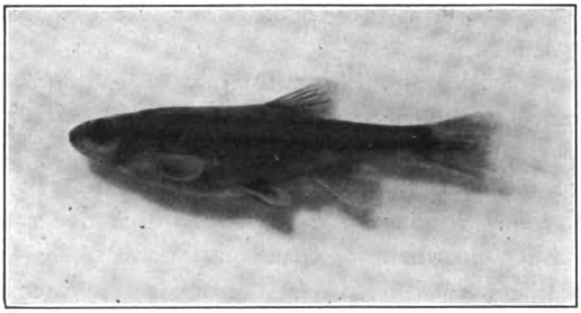

FIG. 2.

FIG. 1, male, and Fig. 2, female specimens of *Chrosomus erythrogaster* Rafinesque, life size.

The males possess "pearl organs" (small conical thickenings of the epidermis), occurring over practically the entire surface of the body, head, tail and fins. They are especially numerous and well developed on the dorsal and lateral surfaces of the body, on the opercula, over the entire surface of the tail, and on the dorsal surfaces of the pectoral fins; they are large, but comparatively few in number, on the dorsal surface of the head. They are much smaller on the ventral surface of the abdomen, and entirely lacking about the mouth and at the tips of the fins.

As seen under the microscope, a pearl organ from the body of a male is a sharply pointed transparent spine, rising from a broad base and pointing obliquely backward, occurring as a thickening of the epidermis over the middle of the posterior margin of each scale. Usually there is only a single pearl organ for each scale, but sometimes two spines occur close together. Smaller rudimentary pearl organs, each consisting of a papilla or thickening of the epidermis without a spine, also occur, one or more over each scale and usually near its center.

The pectoral fins of the male are much larger and stronger, in proportion to size of body, than those of the female. As shown by average measurements, the pelvic fins of the male are also proportionally larger than those of the female, but the difference is not so great as in the case of the pectoral fins.

The females are on the average slightly smaller than the males (see Figs. 1 and 2), but have the abdomen distinctly swollen. There is a small patch of red just back of the operculum. They have the same yellow coloration as the males, but the color is comparatively faint. They possess only rudimentary pearl organs, hardly visible except under the microscope.

A notable exception to the usual sexual dimorphism was found in the case of one unusually large female with well developed ovaries and eggs, but with the secondary sexual characteristics of the male all very well marked.

I had no opportunity to study the color changes and the history of the pearl organs throughout the year.

Sex Ratio.—In order to determine the numerical proportion of the sexes, representative samples of several schools were taken on several different days. The tabulated results are as follows:

Date of Capture.	Mode of Capture.	Number in Catch.	Females.	Males.	Ratio.
1. May 17...............	Hoop net	6	2	4	1 : 2
2. " 29...............	Single sweep of seine	119	9	110	1 : 12.2
3. " 31...............	Hoop net	80	14	66	1 : 4.7
4. June 14...............	Hoop net	15	4	11	1 : 2.7
	Total...............	220	29	191	1 : 6.5

In lots 1, 3 and 4 the sexes were determined by dissection. Since in only one of these specimens the secondary sexual characters were those of the opposite sex, the probable error in determining the sex by the external characters is a negligible quantity. In lot 2 the sexes were determined by the external characters.

These results are sufficient to indicate a decided preponderance in the number of males on the spawning ground at the spawning season, though not necessarily at other times or places.

Spawning Behavior. — The casual observer will find schools of *Chrosomus* occurring in the pools and the deeper portions of the swift water of the brook. On quietly approaching the stream, one will often notice a splashing of the water of the shallow rapids, scarcely distinguishable from the ordinary rippling of the current. Further observation will reveal the fact that in places the water is alive with tiny fishes, struggling and crowding one another in water so shallow that the suface is violently agitated; occasionally a mass of wriggling fishes will flash into view at the very surface, or crowd splashing upon the pebbly shore, where some may be left stranded, later to struggle back into the water.

When alarmed, the fishes almost invariably swim up stream from the shallows. In the deeper water they gradually recover from their fright; those that have hidden amongst the water vegetation or under the shadow of overhanging banks, rejoin the school. Together, moving in unison like a flock of sheep, they surge slowly down stream; but before they reach the shallow water again, they face about with an eddying movement, and swim up stream. This circuit is made repeatedly, the school drifting each time a little farther down stream toward their proper spawning grounds. The behavior of the fishes from the time they are frightened away from the shallows, until they resume active spawning operations on the same ground, forms a series of events which occur on different occasions with unfailing regularity.

On the up stream journey, occasional isolated cases of spawning occur. Several males pursue one female; as the foremost males gain a position alongside the female, the flight and pursuit attain almost lightning-like rapidity. At length two males spawn with the single female as follows: One on each side presses the side of his head against that of the female, all three facing up stream. The two males then crowd laterally against the female, held between them (see Fig. 3); their entire flanks are thus pressed against the sides of the female. While the males are in this position, a rapid vibration of their bodies occurs. The wave of pressure begins at the anterior end of the body and passes backward as a sidewise undulating movement. Other males may attempt to crowd in. So far as observed, the female remains passive. The entire performance occurs so quickly that the details are made out only after considerable practice in observation.

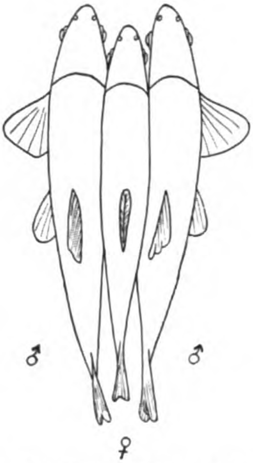

FIG. 3. *Chrosomus* in spawning position. Natural size.

Spawning under these conditions seems to be attended with difficulty. The act of clasping lasts but for an instant; the males seem unable to keep their position after the impetus of their rush up stream is overcome by the current, for swimming movements are necessarily discontinued during spawning. It is doubtful if spawning in the open, without contact with the bottom, is very effective; milt was not extruded in sufficient quantities to be seen. Although the eggs (almost transparent, with light-brown yolk, always difficult to see in the water) could not be seen, it was evident that some were extruded, for immediately after the spawning other fishes poked their noses into crevices between pebbles, just down stream from where the spawning occurred, evidently trying to get at the scattered eggs to eat them.

As the school in its circling movements nears the shallows, a few of the foremost fishes, with shy, frightened movements, dart rapidly down stream through the swift water of the regular spawning grounds; after the first reconnoissance they hurry back to rejoin the main body, and the entire school moves up stream. This is repeated several times; each time the band of more venturesome fishes is increased in number, and a little later, on the up-stream journey, they stop for a time to spawn on the pebbly margin of the stream. Soon the entire school, in a compact body, is spawning on the shallows. With their swift movements and brilliant colors, this writhing mass of several hundred fishes affords a truly remarkable spectacle.

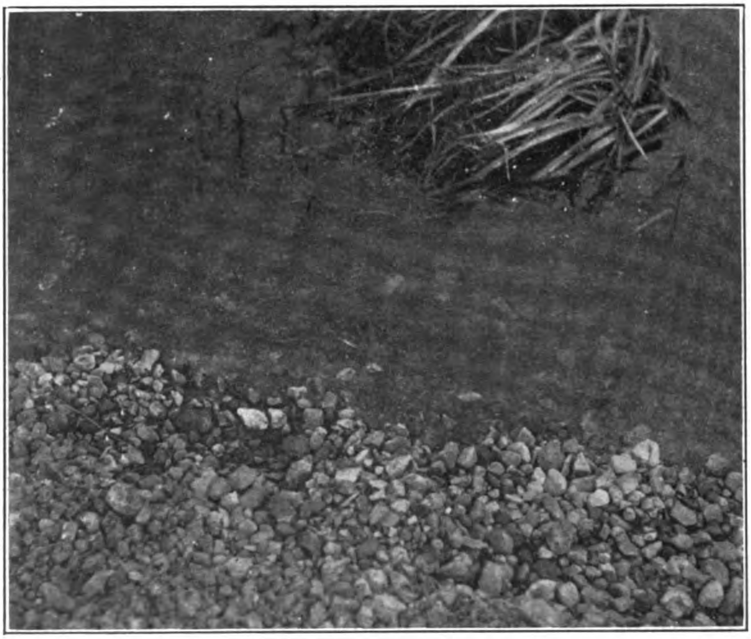

FIG. 4. Spawning grounds of *Chrosomus*. The shallow water near shore is occupied by a school of several hundred *Chrosomus*; some of these may be indistinctly seen, in spawning position, at the surface of the water in the center of the picture.

Persistent attempts to photograph the spawning operations met with poor success, a result due in part to the lack of a suitable camera. Figs. 4 and 5 are reproductions of two of the many views taken.

This procedure of spawning *en masse* in shallow swift water may be regarded as the normal spawning behavior of these fishes. All the fishes face up stream, but their swimming movements are only sufficient to keep them from being swept down stream by the current. In the confused and wriggling mass it is difficult to distinguish the movements of individual fishes; but it may be seen that many males crowd alongside a single female, and those immediately in contact with her may occasionally be seen in the spawning position described as occurring in the deep water.

Gradually the school crowds closer against the shore, rasping over the pebbles and wriggling into water so shallow that their dorsal surfaces are exposed. In these situations small groups become segregated from the main mass; their progress is impeded by the pebbles, so that accurate observation becomes an easy matter. Typically, two males lie alongside a single female; the group becomes wedged in between large pebbles and in close contact with the bottom, so that the males are enabled to keep

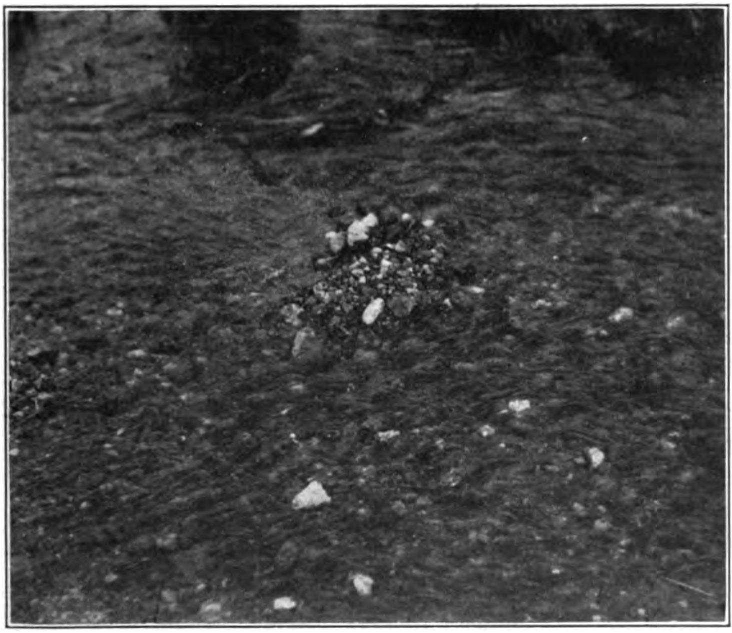

FIG. 5. Spawning grounds of *Chrosomus*. Spawning fishes crowding up on the island of gravel, may be seen near the center of the picture. In the foreground the water is alive with fishes.

their position indefinitely, and lie for many minutes motionless except for the frequently occurring rapid vibration of their bodies. The spawning position is exactly the same as that observed in the open, but the performance is greatly prolonged, and spawning doubtless much more effective. Frequently, groups of four or even more fishes were seen lying alongside, with their bodies in close contact; occasionally a group of six, composed of two trios, each consisting of a single female, spawning with two males.

One marked variation from the usual spawning method was observed. A female in very shallow water spawned with a single male, which crowded her laterally against some pebbles and curved his tail up over her body, thus holding her firmly against the bottom. His body vibrated rapidly and the water became cloudy with milt.

Some further observations on the spawning behavior were obtained by the study of a small school which occupied an abandoned dace's nest. When first observed, this school consisted of a dozen or more males, crowded closely together in the hollow of the nest. Occasional females approached shyly from down stream, singly, or two or three in succession; sometimes darting rapidly, as if excited or frightened, up the middle of the stream; again keeping close to the shore, and occasionally seeking cover. The arrival of a female created intense excitement amongst the males in the nest. They immediately crowded around her, pressing alongside and against her in the hollow of the nest. After spawning, the males devoted considerable attention to prying about between the pebbles as if searching for eggs. Sometimes the males, in a body, left the nest and returned to it repeatedly, moving in an excited manner as if seeking to entice or drive a female into it. The nest was evidently an especially favorable place for spawning.

Eggs in various stages of development were obtained by scooping up the gravel of any of the spawning grounds.

Breeding Season.—The earliest observations were made on May 17. At this time the bright colors of the males were well developed and spawning was in progress, but how long it had been going on is not known. The latest observations were made on June 14; then the schools of fishes spawning on the shallows were small, while in the pools below were larger schools which took no interest in spawning, and were comparatively inactive. The males of these latter schools had nearly lost their bright colors. Evidently the breeding season was nearly over. At this time many of the eggs had hatched out.

During this prolonged breeding season, extending over fully a month, the stream was frequently swollen by freshets, which presumably interfered with the spawning operations.

Discussion. — As shown by the unison of their circling movements when not engaged in spawning, and by the fact that they occur in schools even after the spawning season is over, these fishes possess a strong gregarious instinct. The two conspicuous longitudinal dark stripes on the sides of the bodies of both sexes probably serve as recognition marks.

The distribution of pearl organs over practically the entire body of the male affords a roughened surface which during spawning aids him in keeping his position beside the female. The function of the pearl organs of the Cyprinidæ was first discovered and described by Reighard ('03). The structure of pearl organs has been figured by various writers.

The greater size and strength of the pectoral fins of the male as compared with the female, and the fact that the dorsal surfaces of these fins are unusually well provided with pearl organs, indicate that these fins are of use to the male in holding the female. The male, coming up from the rear, doubtless interlocks his pectoral fin between the pectoral fin and the body of the female, though it was impossible to see this.

The excessive number of males present on the spawning grounds is perhaps correlated with the method of spawning, since two males spawn with a single female.

The observable factors in sex recognition are: the brilliant colors and aggressive bearing of the male; the dull colors, swollen abdomen, and timid, hesitating movements of the female.

It is significant, in its bearing on current theories of secondary sexual characteristics, that the female showing the secondary sexual characters of the male was an unusually large and apparently vigorous specimen; but it would be unwise to base conclusions on a single instance.

The total absence of combat amongst the males accords with the necessity for coöperation in the spawning act. Competition amongst the males is limited to the struggle to get a position next to the female; in this contest the swiftest and strongest succeed.

In general the method of spawning is adapted to fishes with gregarious habits, and would seem hardly likely to be developed amongst fishes whose usual habit of life is solitary.

The pebbles amongst which the eggs lodge afford them some protection, though it appears that some of the eggs are eaten by the parent fishes. The gradual subsidence of the water during the latter part of the season must have left some of the eggs to perish on the shore. That the method of spawning is, in general, a successful one is attested by the abundance of these fishes in this brook.

A comparison with the habits of other inland fishes would be impossible without reference to the unpublished work of Professor Jacob Reighard.

Syracuse University,
April, 1908.

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THE STRUCTURE OF THE SPERMATOPHORES OF AMBYSTOMA PUNCTATUM

BERTRAM G. SMITH

[Reprinted from BIOLOGICAL BULLETIN, Vol. XVIII., No. 4, March, 1910.]

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THE STRUCTURE OF THE SPERMATOPHORES OF AMBYSTOMA PUNCTATUM.

BERTRAM G. SMITH.

Since the recent paper of Wright and Allen ('09) has again directed attention to the breeding habits of *Ambystoma punctatum*, it has occurred to me that a more careful description of the spermatophores than has yet been given might be of value in a comparative survey of the habits of the urodeles. No adequate figures of these spermatophores have yet been published.

The spermatophores of *Ambystoma punctatum* studied by me at Ann Arbor (Smith, '07) were not freshly deposited, and were not in the best possible structural condition for study; hence only a brief description, illustrated by a photograph, was attempted. Since then I have been able to study these structures in abundance under the best possible conditions. As evidence that the spermatophores were freshly deposited, there may be cited the fact that the spermatozoa of some of them were found to be active when examined in the laboratory seven hours after their collection. The results of this study have confirmed the description previously given by me, in regard to distribution and general structure, but have revealed the common occurrence of compound spermatophores, a condition previously unnoticed.

In the early spring of 1909, when the snow had nearly all disappeared, I found specimens of *Ambystoma punctatum* and *A. jeffersonianum*, under rocks on the hillside near "Branchipus Pond," about two miles from the campus of Syracuse University. The study of the habits of these animals was undertaken in order, if possible, to determine by direct observation the exact procedure in the process of fertilization by means of spermatophores in the case of *A. punctatum*, and to obtain data in regard to *A. jeffersonianum*, in which the manner of fertilization is entirely a matter of conjecture.

Conditions made it impossible to keep a very close watch of the pond during the breeding season, and the abruptness of the weather changes caused me to miss the few opportunities for direct observation of the activites of the animals in the field, by means of a lantern at night. Specimens of both sexes were transported to the laboratory and kept under observation in aquaria; several males of *A. punctatum*, and one of *A. jeffersonianum*, deposited masses of seminal fluid on the floor of the aquarium, but nothing resembling the formation of true spermatophores occurred. The largest of these masses was nearly 2 cm. long, and the quantity of sperm deposited by each individual was amply sufficient to form a group of spermatophores. The behavior of the sexes toward each other presented nothing that could be interpreted as normal breeding behavior.

In the pond large numbers of spermatophores were deposited, and these were carefully studied, both *in situ* and in the laboratory; but all the specimens collected were identified, with considerable certainty, as the product of *A. punctatum*. The failure to solve the main problems caused me to lay my notes and drawings aside, awaiting an opportunity to continue the investigation; but the conclusive results of Wright and Allen ('09) have made this unnecessary so far as regards *A. punctatum*.

Identification of Species.—The fact that *A. jeffersonianum* occurs, though less abundantly, in the same region with *A. punctatum* made necessary a determination of the species of the spermatophores found. The only available method seemed to be through a study of the spermatozoa. The material available consisted of spermatozoa from a considerable number of individuals of *A. punctatum*, taken both at Syracuse and at Ann Arbor, and spermatozoa from one specimen of *A. jeffersonianum*, taken at Syracuse. Material from each individual was fixed by a variety of methods, stained, mounted and carefully measured under high powers of the microscope. The average measurements were as follows:

	Acrosome	Head.	Middle Piece.	Tail.	Total Length.
A. punctatum............	20 μ	116 μ	15.0 μ	480 μ	631.0 μ
A. jeffersonianum.........	17 μ	114 μ	11.3 μ	348 μ	490.3 μ

It will be seen that the sperm of *A. jeffersonianum* is in general shorter than that of *A. punctatum*. The middle piece is most easily and accurately measured, and affords the readiest means of differentiating the two species. The range of variation in the length of the middle piece of *A. punctatum* (in several individuals) is $14\,\mu - 16\,\mu$; in *A. jeffersonianum* (in one individual) $11.2\,\mu - 11.9\,\mu$.

The fact that only one individual of *A. jeffersonianum* was considered of course militates against the acceptance of these results as absolutely conclusive; but since material was taken from several individuals of *A. punctatum*, and in all of these there was a decided difference from the results obtained from the single individual of *A. jeffersonianum*, it is highly probable that the species may be distinguished in this way.

Spermatozoa were then taken from a dozen or more spermatophores, each from a different group and presumably deposited by a different individual. This material was treated precisely as in the case of the spermatozoa described above. Somewhat to my surprise and disappointment, all the specimens conformed to the dimensions of the sperm of *A. punctatum*.

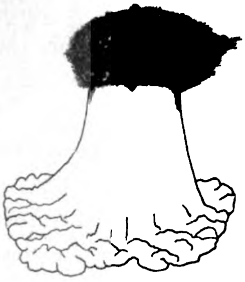
Structure of the Spermatophores.—Two general types of spermatophores are recognizable: simple and compound. The compound spermatophores may be roughly divided into the vertically serial, and the Y-shaped types, the former by far the most common; but both these conditions may be found combined in one compound spermatophore.

A spermatophore of the simple type is illustrated in Fig. 1. It consists of an expanded hummocky base and a stout stalk, of very clear, transparent, gelatinous material, surmounted by a dome-shaped mass of snowy-white seminal fluid.

While to the naked eye the stalk of a simple spermatophore is perfectly clear, under a low power of the microscope it is seen to possess a delicate network of fibrous material. Under high power this is found to be made up of sparsely distributed spermatozoa, interlaced to form a meshwork. In some spermatophores the strands of this network assume considerable regularity. There may be distinguished regular parallel strands extending in the direction of the axis of the stalk, distributed at about equal intervals through its substance; these strands are connected by more irregular cross-strands.

Evidently a few spermatozoa are present in the cloaca at the time when the stalk of the spermatophore is laid down. and these spermatozoa owe the regularity of their distribution to the papillated ridges of the cloaca.

For comparison a drawing (Fig. 2) of a spermatophore of *Diëmyctylus viridescens*, obtained from a specimen in captivity, is placed alongside the figure of the simple spermatophore of *Ambystoma punctatum*. (The spermatophore of *Diëmyctylus* has been previously described and figured by Jordan, '93.) Several spermatophores of *Diëmyctylus viridescens* were available for this study, but none of these differed essentially from the one figured. The average dimensions of twelve simple spermatophores of *Ambystoma punctatum* taken at random, and of two uninjured spermatophores of *Diëmyctylus viridescens*, are as follows:



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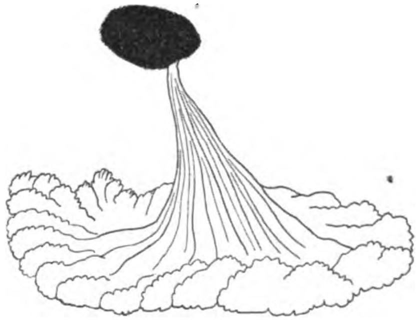

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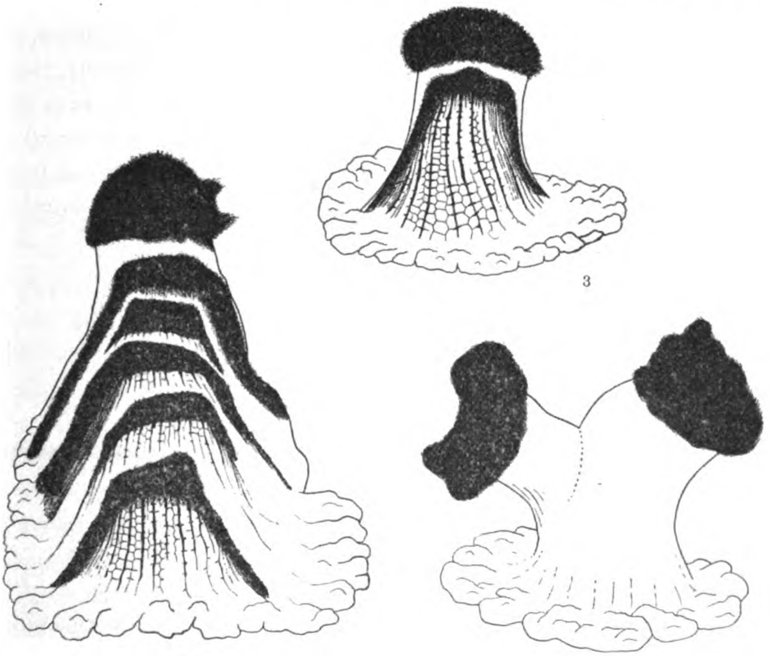
FIG. 1. Camera drawing of simple spermatophore of *Ambystoma punctatum*, × 5. In this and in the following figures the blackened portion is in nature snowy white.

FIG. 2. Camera drawing of a spermatophore of *Diëmyctylus viridescens*, × 5.

	Total Height.	Longest Diameter of Sperm Mass.	Smallest Diameter of Stalk.	Breadth.
A. punctatum.....	6.2 mm.	3.7 mm.	2.5 mm.	6.4 mm.
D. viridescens.....	8.0 "	2.5 "	0.3 "	12.0 "

The simplest and most common case of compound spermatophore is that in which one spermatophore is deposited directly on top of another (see Fig. 3). In the process of deposition of the second spermatophore the sperm ball of the first one is crushed into the form of an inverted cup covering the stalk of the first, and the sides of this cup are stamped with a characteristic pattern by the fluted surface of the cloaca. This pattern reminds one of the fainter meshwork seen with the aid of a microscope in the stalk of a simple spermatophore.

By an increase in the number of spermatophores arranged in a vertical series we obtain the extreme form of the serial type shown in Fig. 4, which is made up of six simple spermatophores.



FIGS. 3, 4 and 5. Compound spermatophores of *Ambystoma punctatum*. All the figures are camera drawings, × 5.

FIG. 3. Compound spermatophore consisting of two simple spermatophores, one superimposed on the other.

FIG. 4. Compound spermatophore of the vertical-serial type.

FIG. 5. Simplest case of the Y-shaped type.

Somewhat rarely, compound spermatophores of the Y-shaped type (Fig. 5) are found. These are apparently formed by the deposition of one spermatophore on the side of the stalk of another. In several cases noted, one arm of the Y-shaped stalk was again compound, consisting of two or more spermatophores in series.

Compound spermatophores are not all so regular in form as those figured. Sometimes successive spermatophores are packed together so closely that is it difficult to distinguish individuals in the complex; but while the first impression obtained from some of these spermatophores was that of great irregularity, closer study showed that all of them might be reduced to the vertical-serial type, the Y-shaped type, or a combination of these.

Some of the sperm masses of the simple spermatophores, or the terminal one in the case of a compound spermatophore, were found considerably frayed as if from being taken up into the cloaca of a female; rarely, the entire ball of sperm was missing. It is of course possible that either condition might be accidental.

In one hundred spermatophores taken at random, the number of each kind was as follows:

1. Simple type	23
2. Compound, two in vertical series	45
3. Compound, more than two in vertical series	21
4. Y-shaped, or combination of this type with (2) or (3)	11
Total	100

In none of the spermatophores examined was there any approach to the complexity of structure shown in European urodeles by Zeller ('05). Zeller's drawings appear to me highly conventionalized.

Discussion.—Of the various kinds of spermatophores of *Ambystoma punctatum*, the simple type is functionally the most perfect; yet even this is not so highly differentiated and structurally so well adapted for its purpose as the spermatophore of *Diëmyctylus viridescens*. In case the of *Diëmyctylus*, I have seen almost the entire ball of sperm removed from the very slender stalk by the female cloaca. In *Ambystoma*, on account of the thickness of the stalk affording a large surface for contact with the mass of spermatozoa, this would be more difficult, and according to the observations of Wright and Allen ('09) does not occur—only a very few sperms, comparatively, are detached and retained by the cloaca.

The only mention of compound spermatophores that I find in the literature is in the case of the axolotl, described by Gasco ('81). While in many cases the spermatophores deposited were single, in one case the male was observed to return repeatedly, at intervals of several minutes, to the same spermatophore and deposit another on top of it, thus building up a compound spermatophore consisting of seven simple spermatophores. Whether the compound spermatophore of *Ambystoma punctatum* is built up in this way I have no means of deciding; it would seem quite as probable that the spermatophores were deposited in rapid succession, while the animal remained in one place — a condition not far removed from what occurs in external fertilization, in which a large amount of seminal fluid is discharged at once. In any case it is difficult to see that any advantage is gained by depositing one spermatophore on top of another and thereby destroying the efficiency of the one previously deposited. As already noted in the case of *Ambystoma punctatum*, the spermatozoa of these spermatophores retain their vitality in water for many hours. The formation of compound spermatophores of the vertical-serial type seems to me a useless and wasteful procedure, and the large proportion of such spermatophores indicates that the spermatophore-forming habit is not highly evolved nor perfectly adapted to its purpose.

In my previous paper (Smith, '07) on the breeding habits of *Ambystoma punctatum*, I ventured the prediction, based on the large number of spermatophores and the manner of their distribution, that the behavior of the adults in the fertilization process would be found to be simpler than in the case of *Diëmyctylus viridescens*. In the latter case only a very few spermatophores are formed by each male, and particular safeguards are necessary in order to insure the delivery of at least one of them to the female cloaca; these safeguards are furnished by the complicated but definite series of acts on the part of the adults which precedes and conditions the deposition of a spermatophore. Just as in the higher vertebrates economy of egg production is correlated with care of the young, so in *Diëmyctylus* economy in the production of spermatophores is accompanied by a considerable amount of certainty as to their fate. This prediction as to the relative simplicity of the behavior of the adults of *Ambystoma punctatum*, the recent observations of Wright and Allen ('09) have abundantly justified. In the light of the observations on the structure of the spermatophores recorded in the present paper, we can go further and say that the entire process of securing fertilization by means of spermatophores is in *Ambystoma punctatum* more primitive, less highly adapted to secure its object with economy of material, than in *Diëmyctylus viridescens.*

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THE NESTS AND LARVÆ OF NECTURUS.

BERTRAM G. SMITH.

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THE NESTS AND LARVÆ OF NECTURUS.

BERTRAM G. SMITH.

THE NESTS.

1. *Nests in a Lake Habitat.*—Through the courtesy of Professor Bennet M. Allen I recently became acquainted with the spawning grounds of *Necturus maculosus* Rafinesque in Lake Monona, Wis. During the latter part of June and the early part of July, 1910, several trips were made to the locality for the purpose of obtaining embryological material.

The "nests" were found in water about 3–5 feet deep and about 50–100 feet from the shore, in a locality where the bottom was strewn with loose flat stones of various sizes. The largest of these stones, about 1½–2 feet in diameter, frequently served as cover for the eggs of *Necturus*. The eggs are attached by the slender stalks of the gelatinous envelopes singly to the under sides of these stones, distributed over an area about 8–10 inches in diameter (see Fig. 1). The presence of minute algæ, etc., in the water made it so opaque that it was impossible to see the bottom; the eggs were obtained by wading in the water, feeling about with the feet for a large flat stone, then bringing it to the surface.

Eycleshymer ('06) describes nests of eggs attached to the under sides of logs, boards, pieces of tin, canvas, etc., but does not mention finding nests under stones. Doubtless any convenient object may be selected as cover.

The number of eggs present in a nest was determined in five cases as follows: 18, 61, 80, 84, 87. The average is 66. The nest photographed contained 84 eggs.

The first embryos were obtained on June 22; these were in an advanced stage of development, with head well formed and a small tail rudiment. The latest embryos were collected on July 5; at this time nearly all the embryos had hatched, only a few unhatched embryos being found in each of several nests. The empty capsules remained attached by their stalks.

Eycleshymer ('06) states: "The time of egg-laying varies in different lakes, depending upon the time when the temperature of the water reaches a certain degree. In the larger, deeper lakes with bold shores this is much later than in those possessing wide shoals. . . . According to Professor Whitman's and my own experience the best time for collecting is during the middle and latter parts of the month of May. The writer has collected eggs as early as May 3, and as late as June 5, but these extremes mark the beginning and closing of the early and late seasons." Unless the dates given are for *newly-laid* eggs, which would

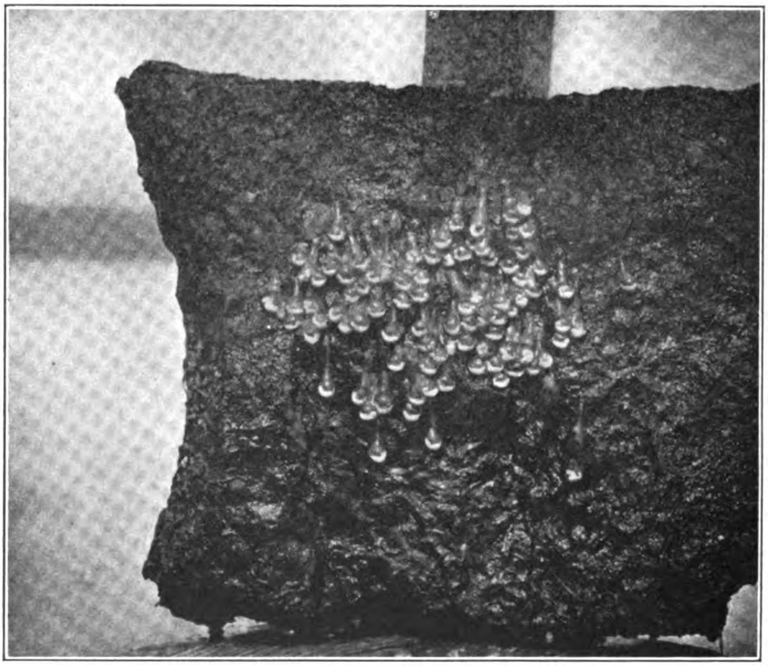

FIG. 1. "Nest" of eggs of *Necturus*. The stone to which the eggs are attached has been removed from the water and set on edge on the wharf; it is about 16 inches in diameter. The embryos are in an advanced stage of development.

hardly be inferred from the text, my experience shows that the collecting season may be much later than Eycleshymer recorded.

A noticeable feature of the development as compared with other amphibians that I have studied, is the uniformity in the stage of development of embryos found in different nests in the same locality. On each of the following dates from four to seven nests were secured: June 22, 25, 29, July 5. On each date all the eggs were found so nearly in the same stage of development that only slight differences could be detected in eggs from different nests. This uniformity points to a very short spawning season—perhaps two or three days—in this locality; it would seem that all the eggs in a restricted area are laid at nearly the same time. But Eycleshymer ('06) says: "The eggs are first deposited in those localities where the water is shallow and exposed for the greater part of the day to the rays of the sun. The period of egg-laying usually covers two or three weeks. There is no foundation whatever for the statement made by Hans Virchow that the animals deposit their eggs so to speak at the same hour."

I had no success in keeping embryos of *Necturus* alive in the laboratory, although later in the year larvæ of *Cryptobranchus* thrived under the same conditions.

2. *Nests in a Stream Habitat.*—I am not aware of any published observations on the nesting of *Necturus* in streams, hence the following notes on the subject may be of interest.

During the late summer and early autumn of the past five years, while searching for adults, eggs and larvæ of *Cryptobranchus*, I have had occasion to overturn numberless stones in the bottom of a large creek tributary to the Allegheny River, in northwestern Pennsylvania. This has resulted in the frequent finding of specimens of *Necturus*.

All the specimens found were small, none exceeding 20 cm. (8 in.) in length and most of them were much smaller. The smallest, taken September 13, 1906, was 35 mm. long (see Fig. 6). This specimen was one of a group of six or seven found under the same stone; the others, aided by the swift current, escaped.

These circumstances led me to suspect that the stream was a spawning ground for *Necturus*. This suspicion received confirmation when, on August 24, 1910, I found attached to the under side of a large rock about fifteen or twenty empty egg capsules of *Necturus*, all in good condition. At the distal end of each capsule was a large round hole through which the embryo had escaped.

THE LARVÆ.

1. *The Embryo at the Time of Hatching.*—Fig. 2 is from a photograph of an embryo of *Necturus* obtained from Lake Monona on July 5, 1910. This embryo was apparently ready to hatch, since nearly all the other embryos in the nest had already hatched out.

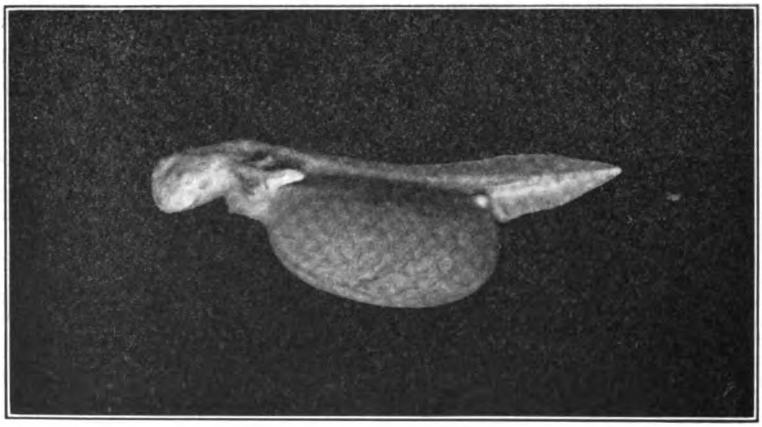

FIG. 2. *Necturus* embryo ready to hatch, killed in Tellyesnicky's fluid and preserved in formalin, July 5, 1910. (× 3½.) From Lake Monona.

The embryos of *Necturus* are hatched in a less advanced stage of development than is the case with *Cryptobranchus* (see Smith, '07, Fig. 9); but in *Cryptobranchus* at least there is considerable variation in the time of hatching and the figure referred to represents one of the more advanced of the newly-hatched embryos, hence the difference may be less than the figures indicate. *Necturus* is hatched in water of a much higher temperature than is the case with *Cryptobranchus*, and this would naturally tend to soften the gelatinous envelope and aid in the early escape of the embryo.

In *Necturus* the large yolk content at the time of hatching is even more noteworthy than in *Cryptobranchus*. Though set free as a larva, the animal is really in an embryonic state so far as its food supply is concerned, until long after its liberation. The only advantages secured by hatching in this condition would seem to be better aeration and opportunities for exercise; these advantages must be in part offset by the increased danger of capture by some larger animal.

Aside from differences in the stage of development, the embryos of *Necturus* and *Cryptobranchus* at about the time of hatching are so much alike that it would be difficult to tell them apart were it not for the noticeably greater bulk of the latter. At the time of hatching the *Necturus* embryo measures about 18 mm. in length, the *Cryptobranchus* embryo about 24 mm.; this difference may be due in part to the more advanced condition of the latter, but throughout the entire embryonic history the size of embryos of corresponding stages is so much greater in the case of *Cryptobranchus* that though eggs from different nests of the same species may vary slightly in size, the extremes of variation of the two species do not overlap.

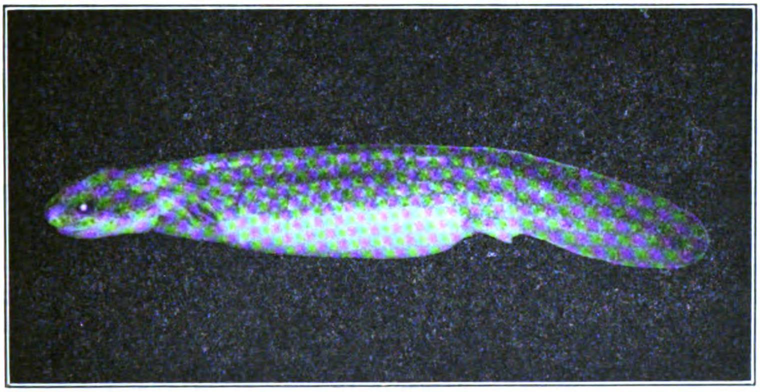

FIG. 3. *Necturus* larva reared from advanced embryo obtained from Lake Monona, and preserved in formalin July 31, 1910. (× 3½.)

2. *Larval Development.*—I have examined a series of early larval stages kindly loaned me for the purpose by Dr. Bennet M. Allen. The series comprises twelve specimens raised from advanced embryos obtained from Lake Monona and preserved in formalin on the following dates: July 31, August 19, 21, 25, 1910.

Specimens preserved July 31 (see Fig. 3) average about 25 mm. long. As compared with *Cryptobranchus* larvæ of the same age after hatching, in *Necturus* the general form of the body is more slender, except that the yolk sac is of relatively greater size. The absolute size of the *Cryptobranchus* larva is much greater. The pigmentation in *Necturus* is less intense, and the general appearance is that of a less advanced stage of development.

These specimens show the beginning of the dorso-lateral stripes, which attain great prominence in later stages and will be described in the stage in which they are most marked.

In the larvæ preserved during the latter part of August, the yolk sac still persists, though its size is so reduced that the abdomen is only slightly distended by it. The lateral stripes are quite distinct, though not so conspicuous as in slightly later stages. The larvæ average about 30 mm. in length. In *Cryptobranchus* larvæ of the same age after hatching, the yolk sac is so reduced that the abdomen is no more distended than in the adult; the color is much darker than in *Necturus*, the form of the body stouter, and the absolute size nearly twice as great.

For a larval specimen 34 mm. long, I am indebted to Mr. L. W. Harrington, formerly an assistant in the Zoölogical Laboratory of the University of Michigan. This specimen was obtained by Mr. Harrington from fishermen in the Detroit River on November 24, 1906, and was examined by me on the same day, before preservation. The transparency of the ventral abdominal wall enables one readily to note that the yolk has been entirely absorbed. Since the coloration conforms accurately to that of the Lake Monona specimens, a description of the color pattern will serve for all the western larvæ examined by me.

As contrasted with the adult, the most striking feature of the 34-mm. larva is the presence of a conspicuous dorso-lateral longitudinal stripe, light yellow in color, on each side of the body. The lateral margin of each stripe is metamerically crenate. The body stripes continue unbroken and without fusion to the tip of the tail; anteriorly, they are separated by a slight break from similar stripes along the margin of the dorsal surface of the head; these latter are sometimes connected at their anterior ends by a faint transverse bar over the tip of the snout.

The ground color of the dorsal and lateral surfaces is a dark brown, with small scattering spots of lighter color especially noticeable along the sides of the body and tail. As compared with the earlier stages the pigmentation is much more intense, but this merely serves to accentuate the light-yellow stripes. It is to be particularly noted that in all the western larvæ that I have examined, the dark color of the dorsal surface of the body is continued along the dorsal edge of the tail between the lateral stripes (see Fig. 4). The ventral surface is pale yellow, almost transparent.

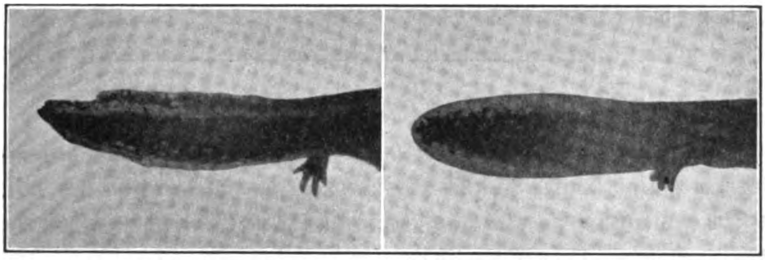

FIG. 4. FIG. 5.

FIG. 4. Caudal portion of a 34-mm. *Necturus* larva taken from the Detroit River, showing color pattern of the tail. The specimen is somewhat shrivelled from partial drying before preservation; in particular the ventral margin of the tail is upturned. Photographed after preservation in formalin. (× 3½.)

FIG. 5. Caudal portion of a 35-mm. larva of *Necturus* taken from a stream in northwestern Pennsylvania, showing color pattern of the tail. Photographed from a formalin specimen. (× 3½.)

Mention has already been made of larvæ taken from a stream habitat in northwestern Pennsylvania. No attempt was made to capture all the specimens found, but a series was preserved representing a gradation in size from the smallest, 35 mm. long, to the largest, 20 cm. in length. Of these the fourteen smallest, all under 15 cm. in length, show larval characteristics in the color pattern.

The smallest specimen, 35 mm. in length (see Fig. 6), was taken on September 13, 1906. This larva is younger and morphologically less advanced than the slightly smaller larva obtained from the Detroit River; but on account of an important difference in the color pattern its description has been deferred.

By dissection it was found that the yolk sac, though reduced almost to the form of a digestive tube, still contained a small amount of yolk. Assuming that this larva had been hatched about 8–10 weeks, we find a similar condition of the yolk sac in *Cryptobranchus* larvæ of the same age after hatching.

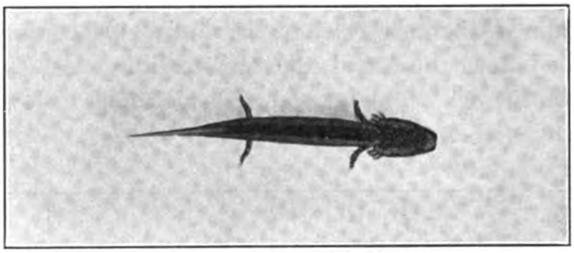

FIG. 6. Living larva of *Necturus*, 35 mm. long. (×$\frac{7}{8}$.)

This specimen differs from the western larvæ in that the dorso-lateral stripes unite in the median line at the base of the tail, to be continued as a single stripe along the dorsal edge of the tail (see Fig. 5). Since this peculiarity is present in all the fourteen larvæ that I have examined from the eastern habitat, it would appear to be a constant difference between the eastern and western forms. Otherwise the color and color pattern of both eastern and western larvæ are the same.

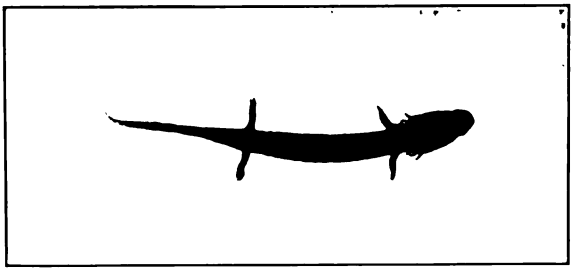

FIG. 7. Larva of *Necturus*, 55 mm. long, photographed after preservation in ormalin. Nearly actual size.

The larger specimens may be described as follows: A 42-mm. larva taken on August 29, 1907, differs morphologically from the 35-mm. larva in its slightly greater yolk content; evidently it is younger, though of greater size. A specimen 55 mm. long

(Fig. 7), taken on August 20, 1906, agrees in its color and color pattern with the 35-mm. larva; the yolk has been entirely absorbed. Another 55-mm. larva taken September 13, 1906, is in the same condition. A 60-mm. specimen taken August 19, 1910, shows a slight loss in the distinctness of the stripes.

The striped pattern characteristic of the larvæ reaches its culmination in specimens of about 55 mm. body length. From this time on it gradually disappears, the light yellow stripes being obscured by dark pigment; during the same period the large black spots, which give the mottled appearance to the color pattern of the adult, become established. With the attainment of a body length of 15 cm. the larval color pattern is entirely replaced by that of the adult. Several specimens with a body length of 20 cm. were dissected and found to be sexually mature.

Eycleshymer ('06), after describing the variation in color of the adult, continues: "It is probable that these variations in color are responsible for a number of specific names. As an instance I might state that some years ago Dr. Garnier ('88) described a small *Necturus*, taken from the Maitland and Lucknow Rivers in Ontario, to which he gave the name *Menobranchus lateralis*, var. *latastei*. 'The coloration above was black, the abdomen sooty and the gular fold white.' During the summer of 1904 the writer was fortunate enough to secure two young animals which measured about 4 and 6 inches respectively. The smaller corresponds closely to the description given by Dr. Garnier and there is every reason for believing that the animal in question is the young of *Necturus maculosus*. The older of the two presents the general coloration of the adult. That *Necturus* should undergo such striking changes in color may appear remarkable to one who has not studied the early stages but when one has followed the changes in color pattern during growth he finds that they are no less striking and remarkable than in the birds."

While I do not doubt the evidence of great variability in the color and color pattern during growth, furnished by those[1] who have reared *Necturus* from the eggs, the fact remains that in all the specimens studied by me not a single non-striped larva has been found, nor is any specimen really black. Moreover, I would point out that Dr. Garnier's description above quoted would make an excellent account of the coloration of a year-old larva of *Cryptobranchus allegheniensis*, having external gills and with a body length of 8 cm.

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[1] In a letter from Dr. Whitman to the writer, dated April 22, 1907, he says: "I have reared *Necturus* from the egg, and I can assure you that now and then a single dark *brown* individual is found among the striped ones. I raised one from the egg, and have had two captured by net. They are rare, but are unquestionably *Necturus* in the cases mentioned."

Zoölogical Laboratory,
University of Wisconsin.

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Reproduced from Vol. IX, Nos. 1-2 (April, 1911), of the Bulletin of the Wisconsin Natural History Society

A CASE OF DEFENSIVE SELF-MUTILATION IN *CRYPTOBRANCHUS*.

BERTRAM G. SMITH.

One of the worst enemies of aquatic vertebrates in captivity is the water-mold, *Saprolegnia*.. Recovery from a severe infection is so rare that this alone would attract attention; but recovery by means of the automatic amputation of infected members is a phenomenon that I have never seen described, and have recently observed for the first time.

During the latter part of November, 1909, seven hellbenders of various sizes were shipped from their native habitat in northwestern Pennsylvania to the Zoological Laboratory of the University of Wisconsin. Placed in an aquarium with shallow running water pumped from Lake Mendota, all but one were promptly attacked with the "nitrogen disease" familiar to fish culturists, and five of them quickly died. The remaining affected specimen displayed remarkable powers of resistance to the disease and, though swollen with bubbles almost past recognition, after three months entirely recovered from this attack.

This specimen, a young adult 35 cm. long, is the subject of the present note. Perhaps because rendered vnunerable by the bursting of the epidermis by nitrogen bubbles, it became infected with *Saprolegnia*. The fungus completely covered the toes of the hind legs, and the last two inches of the tail, but seemed to be confined to these regions.

A crack or fissure, accurately separating the infected from the uninfected portions of the integument, soon appeared; this deepened until it reached the bone, after which the infected extremities of ,the limbs and tail dangled for several weeks connected with the animal only by the bone. The fissures appeared as sharp and clean as if cut with a knife; on one side was healthy living tissue, on the other tissue which seemed insensitive, since the animal did not respond when the affected regions were stimulated with forceps.

On March 15, 1910, the digits of one hind foot had been entirely cast off, and the wound had begun to heal over. The other hind foot had lost the integument and flesh of the first phalanges, and the latter projected as naked bones. The posterior two inches of the tail were still connected with the body by the vertebral column.

About March 30, the infected portion of the tail dropped off, one vertebra being left bare with conspicuously projecting transverse processes.

About three months later (July 7) it was observed that the stumps of the tail and hind feet had entirely healed over, so that the bones were no longer exposed. No signs of infection were visible on any part of the body of the animal. That it had quite recovered its vitality is evidenced by the fact that while handling it I received a bite on the fingers sufficient to draw blood—the only time I have ever been bitten by a hellbender.

About a month later the animal died, apparently from a fresh attack of the nitrogen disease, so that I was unable to observe to what extent regeneration might take place.

The sloughing off of injured or infected tissue is not uncommon in vertebrates but in such cases the tissue is usually gotten rid of piecemeal. In the case described in *Cryptobranchus* the affected portions remained intact until dropped off entire, and this was accomplished by a break in the tissue bordering the region of infection. When we remember that the skin of *Cryptobranchus is* tough and flexible, almost as far removed from being brittle as it is possible to imagine, we find it impossible to explain its breaking at this point as due to purely mechanical factors. Pathological changes in the tissues, proceeding in advance of the actual infection, must so alter their physical properties that a break occurs in precisely the region that is most advantageous to the animal in getting rid of the infection.

Zoological Laboratory, University of Wisconsin.
January, 1911.

NOTES ON THE NATURAL HISTORY OF AMBYSTOMA JEFFERSONIANUM, A. PUNCTATUM AND A. TIGRINUM

BY BERTRAM G. SMITH
MADISON, WIS.

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NOTES ON THE NATURAL HISTORY OF *AMBYSTOMA JEFFERSONIANUM, A. PUNCTATUM* AND *A. TIGRINUM.*

By Bertram G. Smith.

In the spring of 1909, while an instructor in Syracuse University, I undertook a comparison of the spawn and larval stages of *Ambystoma jeffersonianum* and *A. punctatum,* with the object of securing data for the identification of species in these stages, and possibly contributing something of value from a biological point of view. Information as to the specific characteristics would at least be of service to any one using the material for embryological or experimental purposes.

After my departure from Syracuse in the early summer of 1909 it became apparent that I would have no opportunity to study these forms again, and since the results of one season's work were necessarily fragmentary and incomplete, the idea of publishing anything on the subject was abandoned. But the recent article of Piersol ('10), dealing with precisely the same problem, studied under remarkably similar faunal conditions, suggests that taken in connection with his paper my own notes may be of some interest, since in many respects the two accounts supplement each other. I have added some observations on the habits of the adults, and a few notes on *A. tigrinum.*

HABITAT.

Ambystoma jeffersonianum was studied exclusively in "Branchipus Pond" and its immediate vicinity, about two miles south of the campus of Syracuse University. *Ambystoma punctatum* was studied in the same habitat, and several years before in various ponds about Ann Arbor, Mich. *Ambystoma tigrinum* has been observed at Ann Arbor; in the pond on the campus of Lake Forest College at Lake Forest, Ill.; and, rarely, in the pond on "Picnic Point," near Madison, Wis.

"Branchipus Pond" is, in the spring, several acres in extent. On one shore is an almost precipitous hillside, sloping gently at the bottom down to the water line. Near the pond is a shallow ditch, extending at right angles from the hillside. The ditch, and the shore of the pond nearest the hill, are favorite spawning grounds for *Ambystoma.*

Besides the two species of *Ambystoma* mentioned, the following other species of amphibia occur in the locality: *Diemyctylus viridescens, Plethodon erythronotus, Rana pipiens, Rana sylvatica, Hyla pickeringii.* In the early spring, just as the ice is melting from the pond, vast numbers of *Branchipus* sometimes make their appearance; hence the name of the pond.

The hillside mentioned is a steep talus, consisting of rock fragments mingled with vegetable mold. This affords a labyrinth of natural cavities, the ordinary terrestrial dwelling-places of the local urodeles. Here they find abundant food: earthworms, etc.

In the vicinity of Ann Arbor, *Ambystoma punctatum* and *A. tigrinum* breed in different ponds, each species occurring to the exclusion of the other. I have noted only a single bunch of eggs in exception to this rule. The cause of this phenomenon is a matter for conjecture; possibly an explanation lies in an adaptation of the structure of the larvæ (e. g., presence or absence of "balancers") to certain factors in the environment (e. g., character of the bottom, whether leaves, pebbles or mud, etc.); or the larvae of one species may devour those of the other, or monopolize the food supply.

THE SPRING MIGRATION.

The fact that *A. punctatum* winters on land and migrates to water after the opening of spring has been established by Wright ('08). The following observations on this point are in complete accord with Wright's account and point to the same phenomenon in *A. jeffersonianum.*

During the fall of 1908 Branchipus Pond was completely dried up. This was noted in the latter part of September. Since dry weather continued until very late in the fall it seems probable that cold weather overtook the *Ambystoma* before they could have made use of the pond as winter quarters, had it been their habit to do so. The fact that they appeared in considerable numbers the next spring shows that the pond was not necessary as a winter's habitat.

On March 31, 1909, the ice had melted about the edges of the pond. Thorough search was made in the exposed water, and every available cover along the shore line was overturned, but no *Ambystoma* were found. The rocks on the hillside were still frozen fast.

On April 2, the ice had just disappeared from the pond, except some very thin ice that had formed along the shore during the night. Two specimens of *A. jeffersonianum* (male and female), were found under stones at the water's edge. One female specimen of *A. punctatum* was found under a stone 100 feet from water and about 10 feet above water level; this specimen was almost rigid from cold. Only the stones on the slope at the foot of the hill, adjacent to the pond, were now thawed out; the steep hillside, which presumably formed the main winter quarters of the urodeles, was still frozen at the surface. A few days later, when this hillside thawed out, specimens of both species were found in greater numbers in all the localities mentioned, and numerous spermatophores of *A. punctatum* (Smith '07 and '10) appeared in the pond. The animals were evidently nocturnal in their activities, since, in the daytime, only one specimen of each species was ever found in the open water.

Concerning the occurrence of a spring migration in *A. tigrinum* I am in doubt. At Lake Forest specimens were found in the open water as soon as the ice had melted from the edges of the pond. The next day a trap, 60 ft. long, was constructed, consisting of a zigzag paling of burlap extending along one side of the pond; pits were dug at the apex of each angle on the landward side. Though this remained in place until late in the spring, no *Ambystoma* were caught in it. If a spring migration occured at all it must have been extremely early in the season. Hay ('91) remarks: "In Indiana this species appears to pass the winter hiding about the margins of ponds, or in some cases, away from the water, under logs and such places. I have received specimens taken under the ice, in company with *A. microstomum*, in January and February." In the vicinity of the pond on Picnic Point, near Madison, Wis., there is very little cover that would serve for protection during the winter, so well as the matted vegetation and deep mud of portions of the pond itself. It seems quite probable that, in the case of *A. tigrinum* at least, migration is an event depending somewhat upon ecological conditions.

THE SPAWNING SEASON.

As shown by the study of the two species of *Ambystoma* occurring together in Branchipus Pond, the spawning season of *A. jeffersonianum* is distinctly earlier than that of *A. punctatum.*

In 1909 the first eggs of *A. jeffersonianum* were found on April 5; the first eggs of *A. punctatum* on April 13. The precise duration of the spawning season was not determined, but no fresh eggs of either species were found after April 16.

At Ann Arbor newly-laid eggs of *A. punctatum* were found on April 1, 1905, and April 9, 1906. I have no records for first appearances of eggs of *A. tiginum* in this locality, but my general impression is that the spawning season is slightly earlier than for *A. punctatum.* At Lake Forest the first record of eggs for *A. tigrinum* in 1907 is March 19, and the last for newly-laid eggs is April 4, the spawning season having been interrupted by a week of cold weather. At Madison in 1910 the first eggs of *A. tigrinum* were found on March 26; they were in early segmentation stages.

THE ADULTS.

Ambystoma jeffersonianum (see Plate I, Fig. 1) is usually covered with small white spots, irregular in size and distribution; in some specimens these are almost entirely absent.

In its natural habitat, *A. punctatum* (see Plate II, Fig. 3) has spots of a brilliant yellow; in captivity, exposed to light, the color fades to a dull yellow; in formalin it is changed to a dull gray.

When captured in the early spring, and kept for a few days in confinement, all three species shed the cuticle, usually intact or nearly so. The most perfect specimens of entire shed cuticles were obtained in 1907 from *A. tigrinum.* Four shed cuticles were obtained from the same number of specimens in captivity, and of these three were intact, one broken into two pieces. All were turned almost completely wrong-side out. The cuticle is

PLATE I.

AMBYSTOMA JEFFERSONIANUM.

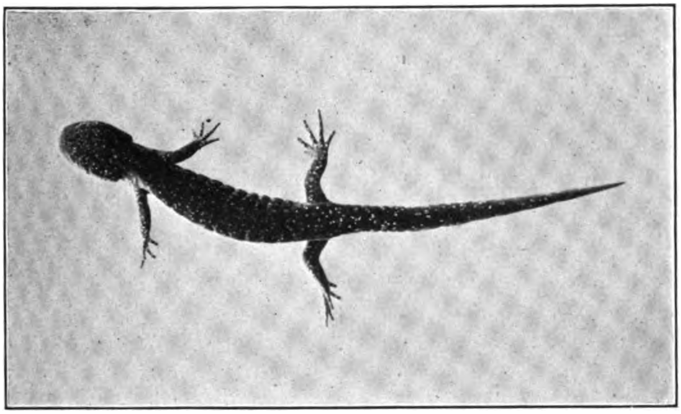


Fig. 1. Adult specimen, living, one-half natural size.

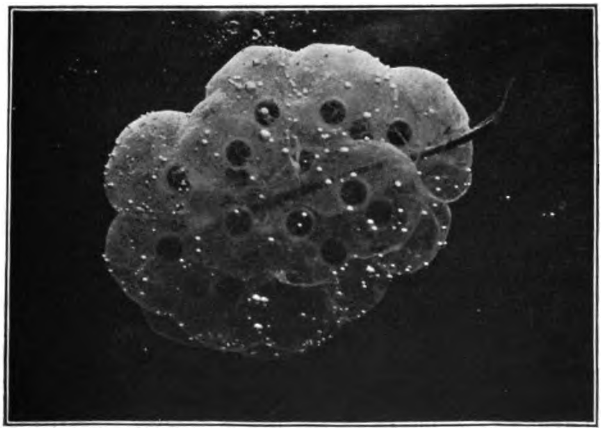


Fig. 2. **Egg mass, natural size, living. The light spots are air bubbles which are normally present in the gelatinous envelopes.**

first loosened at the lips, and then rolled backward, aided by locomotor movements of the animal; the cuticle of the feet comes off like a glove.

In *A. jeffersonianum* and *A. punctatum* the cuticle was usually broken into two or three pieces. The process of shedding, observed when just begun, was found complete 2-3 hours later. Each specimen evidently sheds the cuticle at intervals of a few days.

When handled, individuals of both *A. jeffersonianum* and *A. punctatum* often become immovable. Two one-year-old specimens of *A. punctatum* (see Plate 2, Fig. 3), active when captured, were brought to the laboratory and placed in water in a finger-bowl at room temperature; so far as could be judged from repeated though not continuous observation, they kept the same position for 24 hours. When first handled at the expiration of this time, they were thought to be dead; after being handled for several minutes, they slowly showed signs of life and normal activity. Perhaps this was a "death feint" or hypnotic state.

By daylight, in the aquaria, *A. jeffersonianum* was more active than *A. punctatum.* When watched at night by electric light,*A. punctatum* was more active, seemingly in efforts to escape.

In captivity both species will eat earthworms. *A. jeffersonianum* will eat readily even when newly captured; recently captured specimens of *A. punctatum* refused this food.

SPERMATOPHORES, AND THE TIME OF FERTILIZATION

The distribution and structure of the spermatophores of *A. punctatum* have already been adequately described (Smith '07 and '10), except for the trifling correction that the spermatophores are sometimes deposited on a vertical as well as a horizontal surface. The breeding behavior of *A. punctatum* has been described by Wright and Allen ('09).

The deposition of spermatophores by *A. punctatum* precedes egg-laying by several days. In 1909 the first spermatophores were found on April 5, the first eggs of *A. punctatum* on April 13.

In Branchipus Pond all the spermatophores studied were identified as belonging to *A. punctatum.* In ponds inhabited by *A. tigrinum* I have been unable to find spermatophores of *Ambystoma;* observations have been confined to the spring. The extremely early spawning season of *A. jeffersonianum* and *A.*

tigrinum suggests the possibility of an autumnal fertilization. In this connection it may be mentioned that the testes of an adult *A. tigrinum,* preserved Sept. 21, 1907, were very large and swollen with ripe or nearly ripe spermatozoa, while an adult of the same species, taken March 24, 1910, contained no sperm that could be obtained by stripping, and when preserved April 3, 1910, the testes were extremely small as is usual in urodeles after the breeding season. Sections showed the testes of the latter specimen to contain very few spermatozoa, and those in a state of degeneration.

THE EGGS AND THEIR ENVELOPES.

In all three species the eggs, with their individual gelatinous envelopes, are aggregated in masses by means of a common jelly envelope, which varies greatly in thickness in the different species.

I have identified the eggs of *A. punctatum* by obtaining them from a female in captivity; eggs of *A. tigrinum* through the exclusive occurrence of this species at Lake Forest; and eggs of *A. jeffersonianum* through a process of exclusion.

In twelve clusters of eggs of *A. punctatum* the average number of eggs in a cluster was found to be 56, the range 30-95. Clarke ('79) states that the masses "vary in size from a small bunch of three or four eggs to a large mass containing two hundreds eggs and weighing sixteen ounces." According to Wright and Allen ('09), the number of eggs in a complement varies from 130 to 225; these may be deposited in one to ten bunches, two or three per female being a fair average.

In 52 egg masses of *A. jeffersonianum* the average number of eggs was found to be 14, the range 1-35. Piersol ('10) states: "The typical spawn mass of *A. jeffersonianum* is a small one, the number of eggs being usually about twenty; the extremes encountered have been small masses of jelly without any eggs, and a mass containing forty-one. The complement of ripe ovarian eggs carried by two females of average size was 128 and 161."

My notes contain only two records of the number of eggs in the egg masses of *A. tigrinum*—bunches of 53 and 75 eggs respectively.

The egg masses of *A. punctatum* (see Plate II, Fig. 4) average much larger in size than those of either *jeffersonianum*

PLATE II.

AMBYSTOMA PUNCTATUM.

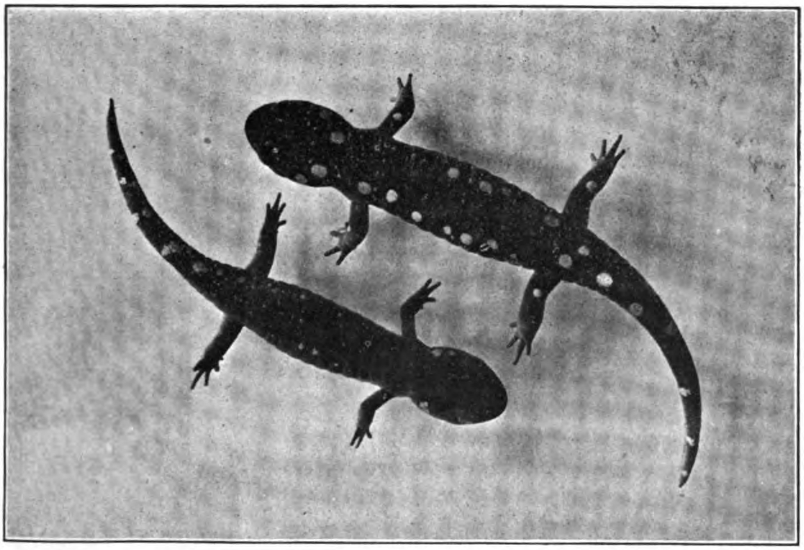


Fig. 3. One-year-old young, natural size, formalin specimens.

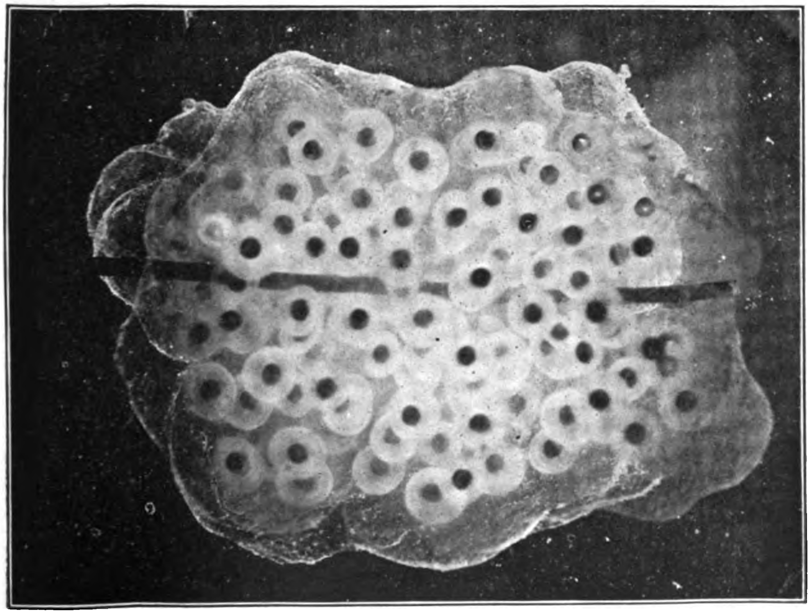


Fig. 4. **Egg mass, natural size, preserved in formalin.**

(Plate I, Fig. 2) or *tigrinum.* This greater size is due not so much to the number of eggs present—for this may be as great in *tigrinum* as in *punctatum*—as to the greater thickness of the common jelly mass. The photograph does not differentiate the common gelatinous envelope of *A. jeffersonianum,* which is extremely thin.

As a result of these differences in the thickness of the common gelatinous envelope we find decided differences in the contour of the egg masses of the three species. In *A. punctatum* the outlines of the individual egg envelopes are obliterated in the general contour; in *A. jeffersonianum* they are almost perfectly preserved, the entire cluster bearing some resemblance to a bunch of grapes. In other words, the eggs of *A. jeffersonianum* form a much *looser* cluster than those of *A. punctatum.* The egg masses of *A. tigrinum* occupy in this respect an intermediate position.

A further distinction between species is that the egg masses of *A. punctatum* possess in general a greater degree of rigidity and firmness, due perhaps to a denser consistency, perhaps to the greater bulk, of the jelly. In this respect also, *A. jeffersonianum* presents the opposite extreme, *A. tigrinum* occupies an intermediate position.

The eggs of *A. punctatum* and *A. tigrinum* are aggregated more closely, forming a more compact group, than those of *A. jeffersonianum.*

The eggs of *A. punctatum* average slightly larger than those of *A. jeffersonianum.* There seems to be greater variation in the size of the latter. The range of the diameter of the two species apparently overlaps. Accurate measurements are desirable as a basis for the comparison.

In the egg masses of *A. jeffersonianum* there is often observable an arrangement of the eggs in from two to three spirally arranged parallel rows. In *punctatum* I have found no such tendency.

The eggs of all three species are invariably attached to some object—the stems of plants, leaves, twigs, etc. In the natural environment the presence of air bubbless in the jelly mass is a normal occurrence; they are shown in the photograph of the egg mass of *A. jeffersonianum.*

HISTORY OF THE EGGS BEFORE HATCHING.

During the early stages of development the space filled with liquid surrounding the individual egg is small, but in the later stages it enlarges to keep pace with the growth of the embryo.

I have searched in vain for spermatozoa imbedded in the egg envelopes of *A. jeffersonianum* and *A. punctatum*.. Clarke ('79) says of *A. punctatum* breeding in captivity: "The eggs were found to have adhering to their outer shells a considerable number of these male elements, but I could not find, after trying a great many times, any spermatozoa within even the outer shell." So far as this evidence goes, it would seem that in the process of internal fertilization the spermatozoa ascend the oviducts and reach the eggs before the envelopes are laid down. But Kingsbury ('95, p. 290) says: "I have never seen zoosperms in the oviducts of any species sectioned by me, in almost all of which the lower portion of the oviduct was examined." In *Cryptobranchus,* in which fertilization is external, spermatozoa are always to be found imbedded in the egg capsule and floating in the fluid-filled space within.

I have found polyspermy occurring in the eggs of *A. tigrinum*. This point will be discussed in a later paper. I have not yet studied the fertilization stages of the other species.

The low vitality in the eggs of *A. jeffersonianum* reported by Piersol ('10), resulting in the loss of about three-fourths of the eggs before gastrulation, has not been evident in the material studied by me.

The approximate time record, not quite complete, for the two species, follows:

Stage.	*A. jeffersonianum.*	*A. punctatum.*
Crescentic blastopore ..	**.....................**	**50 hrs. after fertilization.**
Neural groove	**22 hrs. after crescentic blastopore.**	**30 hrs.**
Closing neural folds...	**16 hrs.**	**20 hrs.**
External gill rudiments	**33 hrs.***	**.....................**
Hatching	**7—13 days.**	**12—22 days after closing neural folds.**
Total time to hatching.	**10—16 days after crescentic blastopore.**	**16—26 days after fertilization.**

From this table it will be seen that the time required for development to hatching is greater in the case of *A. punctatum,* and that even in the early stages the development is slower. Hence the longer time in the case of *A. punctatum* is not due entirely, if at all, to the greater thickness of the gelatinous envelopes.

Embryos of *A. tigrinum* reared at Lake Forest required for hatching a period of from 18 to 20 days.

LARVAL HISTORY.

Larvae of *A. tigrinum* were reared at Ann Arbor in 1906 and at Lake Forest in 1907; *A. punctatum* was reared at Ann Arbor in 1906 and at Syracuse in 1909; *A. jeffersonianum* was reared at Syracuse in 1909. In each case the larvæ were kept in good condition for a little over a month after hatching, circumstances preventing my caring for them for a longer period. The larvae were studied in the living conditon and series preserved for future reference. The following account deals more particularly with the larvae studied at Syracuse.

An effort was made to keep the conditions for the two species, *jeffersonianum* and *punctatum,* practically alike, in order to avoid possible errors of comparison from the occurrence of modifications due to environment, such as are recorded by Powers ('07). The larvae of both species were fed at first on entomostraca—chiefly *Cyclops* and *Daphnia*.. But soon after *A. punctatum* hatched the supply of entomostracans failed almost entirely. This diet was then supplemented by scraped raw beef fed every two or three days to those larvae that would take it. In all cases the larvae took no notice of the meat unless it was moved. *A. jeffersonianum* readily ate the meat, and not satisfied with this, about eight days after hatching commenced to eat each other; the unequal rate of growth facilitated this cannibalistic practice. Ordinarily, one larva partly swallows another nearly as large as himself, digests a portion of the body, then regurgitates the rest. One larva was found swallowed, tail first, nearly to the eyes, by another less than twice his size. The result of this practice is that in time usually only one larva is left in each aquarium.

For a long time *A. punctatum* refused meat and apparently ate nothing, except perhaps the scanty supply of entomostracans. Cannibalism was not noted in *A. punctatum* during the five weeks they were kept under observation. At the end of five weeks only the largest specimens would take meat.

A striking morphological feature common to the early larvae of both *jeffersonianum* and *punctatum,* but absent in *tigrinum,* is the presence of "balancers" at the sides of the head (see Plate III, Figs. 5, 6, 7, 9, 10, 11). These balancers are sticky at the tips, and serve to keep the larva erect while resting on a solid surface. About two weeks after hatching the balancers degenerate and disappear; by this time the broadening of the head, and growth of the anterior limb rudiments, makes them unnecessary. Hay ('91) cites Hoy ('71) to the effect that at the time of hatching the larvae of the "tiger salamander" possess short "holders," which are lost ten days later. This would seem to be a mistake in the identity of the species. The absence of balancers is a characteristic sufficient to distinguish the larvae of *tigrinum* from those of *jeffersonianum* and *punctatum.*

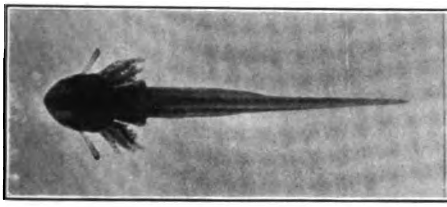
The most marked difference between the newly hatched larvae of *jeffersonianum* and *punctatum* is in the color pattern. Yellow and black pigment are present in both species, and in *A. jeffersonianum* there is a tendency toward the segregation of the black pigment along the sides into a row of 4-6 large black spots, shown but faintly in the photographs (see Plate III, Figs. 6 and 7). These spots vary greatly in distinctness in different specimens, but are always present in a considerable proportion of them, and since they are absent in *punctatum,* afford a convenient character for differentiating the species. The spots sometimes persist for a month after hatching.

The newly-hatched larva of *A. Jeffersonianum* is slightly stouter in body form than that of *A. punctatum.* The latter is hatched in a slightly more advanced stage of development.

A synoptic comparison of the larvae of the two species follows (see also Plate III). The body length after hatching is a character too variable for consideration in this connection.

PLATE III.

A. JEFFERSONIANUM. A. PUNCTATUM.



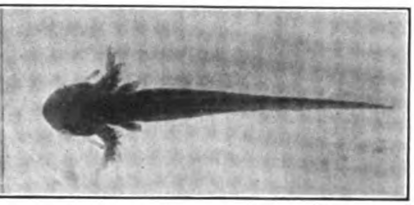
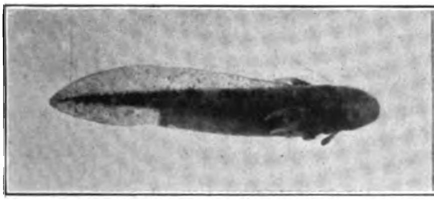


Fig. 5. **Newly hatched, living, X4**

Fig. 9. **Newly hatched, living, X4.**



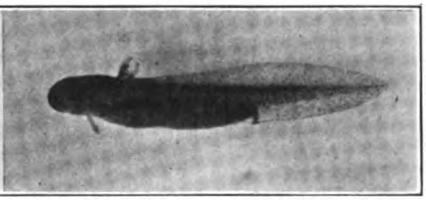
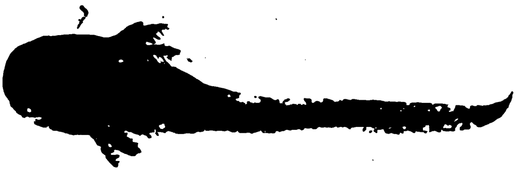


Fig. 6. **Newly hatched, formalin specimen, X4.**

Fig. 10. **Newly hatched, living, X4.**



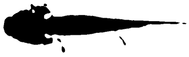
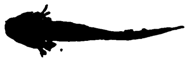


Fig. 7. **Eight days after hatching, formalin, X4.**

Fig. 12. **One month after hatching, life size, living.**



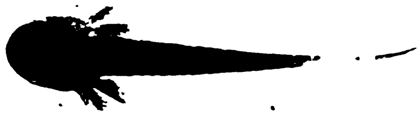


Fig. 8. **One month after hatching, natural size, living.**

Fig. 11. **Eight days after hatching, living, X4.**

A. CONDITION AT TIME OF HATCHING.

Characters.	*A. jeffersonianum.*	*A. punctatum.*
Length	11 mm.	12-13 mm.
Form of body	Stout.	More slender than in *A. jeffersonianum* and tail forms a greater proportion of the entire length of the body.
Balancers	Sometimes not well developed at the time of hatching, but elongate very rapidly and in a day or two attain their full development.	Well developed at the time of hatching.
Eyes	Sometimes covered with opaque integument, but this disappears in a day or two.	Usually functional.
Color and color pattern	Yellow and black pigment, the latter often segregated along the sides to form 4-6 large dark spots.	Both yellow and black pigment, but no marked grouping to form large spots.
Front limb rudiment	Slightly developed.	Well developed, two digits.

B. EIGHT DAYS AFTER HATCHING.

Characters.	*A. jeffersonianum.*	*A. punctatum.*
Balancers	Present.	Present.
Color pattern	Usually spots, as described.	No spots.
Front limb rudiment	Elongated, sometimes two digits.	With two digits.

C. SEVENTEEN DAYS AFTER HATCHING.

Characters.	*A. jeffersonianum.*	*A. punctatum.*
Balancers	Lost (about 14 days after hatching).	Lost (15-18 days).
Color pattern	Spots sometimes present.	No spots.
Front limb rudiment...	With three digits.	With four digits.
Hind limb rudiment...	Beginning, no digits.	Beginning.

D. ONE MONTH AFTER HATCHING.

Characters.	*A. jeffersonianum.*	*A. punctatum.*
Form of body.........	Usually stouter than *punctatum.*	Usually more slender than *jeffersonianum.*
Color pattern	Spots sometimes persist.	No spots.
Front limbs	Well developed.	Well developed.
Hind limb rudiments...	Much variation in size and degree of development.	Much variation as in *jeffersonianum.*

Month-old specimens taken from their natural environment are usually much further advanced than those of the same age reared in the laboratory.

I have had no opportunity to rear larvae to a greater age than five weeks, but have captured specimens from the aquatic habitats of all three species at various dates during the summer. I feel confident that, in the light of the experience gained with earlier stages, these larvae can easily be assigned to their proper species.

The late larval stages of *A. tigrinum* have been described and figured by Powers ('07), with particular attention to the variation in form due to different environments. All the larvae that I have classified as *A. tigrinum* show a characteristic tadpole-like form of body, with large broad head and rapidly tapering body. In some cases there can be no doubt as to the species, there being no other in the locality. *A. punctatum,* on the contrary, has a narrow head and long slender body, more like the adult. Some of the variations of *A. tigrinum* figured by Powers look to me almost exactly like normal specimens of *A. punctatum.* With respect to body form, *A. jeffersonianum* is intermediate between *A. tigrinum* and *A. punctatum.*

The color of *A. punctatum* larvae varies considerably with the habitat, being very dark in shaded pools containing dead leaves and much organic matter in solution; lighter in more open situations. In a larval specimen, presumably *A. punctatum,* captured August 2, 1906, a row of sharply defined small light spots occurs along the upper part of the sides of the body, very much as in the adult though situated not quite so far dorsally. In a larval specimen, presumably *A. jeffersonianum,* taken from Branchipus Pond on June 14, 1909, the row of four or five large dark spots on the sides of the body, noted in the earlier stages, was still clearly defined.

In all the late larval stages examined the external gills were in a well-developed condition, showing no signs of the degeneration accompanying the metamorphosis. Some young post-larval specimens of *A. punctatum* (see Plate II, Fig. 3), captured in April, 1909, were not much larger than the largest larvae observed in August, hence were probably one year old.

I have refrained from figuring these later stages, since their identity has not been established beyond the possibility of a doubt by rearing them from the egg.

Zoological Laboratory, University of Wisconsin.
January, 1911.

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The Embryology of Cryptobranchus Allegheniensis

Including Comparisons with Some Other Vertebrates

Part I

Introduction; the History of the Egg before Cleavage

BY

BERTRAM GARNER SMITH

Submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy, in the Faculty of Pure Science, Columbia University

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I. INTRODUCTION; THE HISTORY OF THE EGG BEFORE CLEAVAGE

BERTRAM G. SMITH

From the Zoological Laboratory of Columbia University

FIFTY-SIX FIGURES

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I. INTRODUCTION

For more than a generation zoologists have eagerly sought for the embryological material of the hellbender, Cryptobranchus allegheniensis Daudin. Until quite recently these efforts have been conspicuously lacking in success. It seems remarkable that the life history of an animal so large, so abundant in localities easy of access, and so important from a phylogenetic point of view, should so long remain shrouded in mystery. But the same difficulty has been encountered in attempts to work out the natural history of several nearly related forms. Eycleshymer ('06) says:

> After years of persistent and patient effort Professor Whitman finally discovered the nests and eggs of Necturus. Only those who have for years been baffled in their attempts to obtain the embryological material of other North American urodeles, such as Siren, Amphiuma, and Cryptobranchus can properly appreciate the enormity of the task.

In the case of Cryptobranchus the difficulty in finding embryological material seems to have been enhanced by the unusual breeding season of the animal; the eggs are laid in the fall, while most amphibia spawn in the spring. Townsend ('82) published a general description of some fertilized eggs which he states were deposited in August. McGregor ('96) described very briefly an embryo 16 mm. in length, and ('99) stated that the eggs are deposited in August and September. Yet the information thus acquired in regard to the time of spawning seems not to have become generally known to others who were searching for the eggs. A suggestion might have been obtained from Sasaki's ('87) observation that the Japanese 'giant salamander,' Cryptobranchus japonicus (Megalobatrachus maximus Schlegel), deposits its eggs in August; but this also seems to have been overlooked. Reese ('04) succeeded in obtaining some unfertilized eggs, of which he gave the first detailed description.

The embryological record for Cryptobranchus allegheniensis remained almost a blank until 1906, when I published a preliminary report containing, besides a description of the sexual elements, a brief account of the external development during the cleavage stages. A later contribution (Smith, '07), devoted chiefly to the habits, more particularly the breeding habits, included a very general account of the life history.

From a phylogenetic point of view great interest attaches to the amphibia; there is no doubt that they lie close to the extinct ancestral stock of the highest forms of vertebrate life. Concerning the origin of the amphibia themselves Kingsley ('99) says: "All the facts of structure and development go to show that the amphibia have arisen from the crossopterygian ganoids, and that existing groups have descended from the stegocephali, each by its own line of ancestry." But when we inquire further, and attempt to trace more particularly the origin of any group of existing amphibia from an extinct form exhibiting affinities to the crossopterygii, we are landed at once in the midst of uncertainties. Confining our attention to the urodeles, we are confronted with the difficult question of the phylogenetic relationships of the different members of this group. The problem will be more fully discussed in a later section; for the present it will be sufficient to call attention to one of its leading aspects. From existing urodeles we may select a series of forms illustrating all stages in a transition from an aquatic to a terrestrial mode of life, or vice versa. In which direction should the series be read? Or have we stated the question incorrectly, and have the urodeles reached their present condition, some from an aquatic, some from a terrestrial ancestry?

In studying this aspect of the phylogenetic problem our attention cannot fail to be attracted by Cryptobranchus. For here we have a urodele whose entire life is spent in the water, characterized by persistent gill slits, the most primitive brain (Osborn, '88), and external fertilization (Smith, '07). On the other hand Cryptobranchus is known to possess deciduous external gills, functional lungs, and a method of locomotion by crawling on the bottom which suggests a former terrestrial habit. Is Cryptobranchus primitively aquatic, or does it come down to us bearing evidence of a former land-living existence? An answer to this question would go far in advancing our knowledge of the phylogeny of the entire group.

In the solution of our phylogenetic problem comparative anatomy, paleontology and embryology must work together. It is the embryological evidence that has hitherto been most conspicuously lacking. Notwithstanding the important position of the aquatic urodeles, it is here that we find one of the widest gaps in our knowledge of comparative embryology. Not only has the development of Cryptobranchus allegheniensis remained undescribed, but little or nothing is known concerning the embryology of most of its near relatives. Very recently, it is true, considerable progress has been made in the study of the embryology of Cryptobranchus japonicus, but part of this work was done on very scanty material, and the field is by no means exhausted. Of the development of Amphiuma and Siren practically nothing is known. Some results have been obtained with special problems in the development of Necturus, but the life history has not been covered in a comprehensive manner. For a study of the phylogenetic relations of these forms a knowledge of the development in its general aspects as well as along special lines is imperative; and in no other form do the embryological data promise to shed greater light on phylogenetic problems than in the case of Cryptobranchus.

For the analysis of developmental processes from a morphogenetic point of view the eggs of Cryptobranchus present certain favorable features. One of these is the presence of a larger amount of yolk than is known in the egg of any other amphibian; they are thus favorable objects for the study of the influence of yolk on development. The eggs, moreover, are lacking in pigment, and the early segregation of the yolk gives a translucency to parts of the embryo even in the gastrula stage, enabling one to study satisfactorily in the living egg some of the internal features of development. The embryo is found to respond admirably to the influence of chemicals modifying the course of development; for certain experiments of this sort it gives results decidedly more definite than have been obtained with the embryo of the frog.

The present contribution is to be followed by other parts dealing with the embryonic and larval development.

This investigation has been pursued under a great variety of circumstances, and with many protracted interruptions due to the pressure of other work. Field work on the habits of Cryptobranchus, the collection and preservation of material, and the study of the living egg, have been carried on each autumn ('05–'11 inclusive) in northwestern Pennsylvania. For comparison with Cryptobranchus, I have collected embryological material of Necturus during the seasons of 1910 and 1911, from Lake Monona, Wisconsin. Laboratory work, principally on preserved material, begun in the Zoological Laboratory of the University of Michigan ('05–07), has been continued in the zoological laboratories of Lake Forest College ('07), Syracuse University ('08–09), the Bureau of Fisheries at Woods Hole (summer of 1908), the University of Wisconsin ('09–11), and Columbia University ('11–12). To the directors of these respective laboratories I wish to express my sincere thanks for uniform courtesy in placing the resources of each institution at my disposal. To Professor Bashford Dean, under whose direction the work is being carried on during the present year, I am profoundly indebted for his constant encouragement, kindly criticism, and valuable advice; for this I desire to record my grateful appreciation.

II. THE ADULTS

A. HABITAT

Cryptobranchus allegheniensis was found abundantly in the Brokenstraw Creek, a tributary to the Allegheny River, in northwestern Pennsylvania. The most favorable locality extends from the confluence with the Allegheny five or six miles upstream. The stream has a rather rapid descent, and a gravelly or rocky bottom. Shallow and rocky rapids make up the greater part of its course, alternating with areas of deeper and more quiet water.

As a rule, Cryptobranchus is found more abundantly in rather shallow and rapid water, where large flat rocks afford suitable cover. Usually the animals lie concealed in cavities under these rocks. As more than one individual is seldom found under a single rock, we conclude that its life is in general a solitary one. Cryptobranchus rarely comes out of its hiding place in the daytime, except in the spring and early summer and during the breeding season (the first two weeks of September). At night they venture abroad in large numbers; they are then seen by fishermen spearing by torchlight, who commonly report them in the deeper and more quiet water.

The cavity or cavern used for a more or less permanent dwelling-place has a rock for its roof and the gravelly bed of the stream for its floor. In perhaps the majority of cases, ready-made cavities are chosen as homes, and these are reached by a natural opening. But the cavity sometimes bears evidence of having been in part hollowed out by the animal, and is occasionally reached by a single tunnel-like entrance on the down-stream side of the rock; this is more often the case in cavities used for spawning purposes.

There is a striking similarity between the habitat of Cryptobranchus allegheniensis and that of the 'giant salmander' of Japan as described and figured by Ishikawa ('04).

B. GENERAL CHARACTERISTICS

1. Size

Out of the many hundreds of adults captured, the largest male (September 3, '08) measured 60 cm. (23½ inches) long and weighed 2½ pounds. The largest female captured (September 3, '09) weighed exactly 3 pounds. The latter animal unfortunately escaped from the aquarium in which it was confined and was not measured; probably it was no longer than the longest male, but heavier because distended with eggs. Professor McGregor reports a specimen 25 inches long, taken from the Scioto River.

The great majority of specimens captured by me were much smaller; specimens of about 30 to 50 cm. were most frequently taken. The smallest sexually mature male measured 30 cm.; the smallest mature female 35 cm.

2. Form

As compared with the young, the adult is more flattened dorsoventrally—an adaptation to life in shallow crevices. The head particularly shows this flattening: it is wedge-shaped as viewed from the side, a form which enables the animal to force its soft body into very shallow crevices.

Moreover, as compared with the young, the adult is distinguished by a general looseness and wrinkling of the skin at the sides of the body, forming broad lateral horizontal folds; and by similar flaps of skin on the posterior sides of the limbs. During locomotion these folds and flaps undulate in the water, contributing to the uncouth appearance of the animal.

3. Coloration

Young sexually mature individuals vary little in color or color pattern. The ground color is dull brown, with conspicuous black spots and less conspicuous yellow spots scattered over the dorsal and lateral surfaces. Both kinds of spots are irregular in size and form. The coloration of young adults is practically that of immature specimens from 16 cm. body length upwards; in these stages the spots are more conspicuous than in the larvae or the older adults. In the older, full-grown specimens the general color effect may vary in two ways: it may become either greenish-brown or decidedly reddish brown. As stated by Reese ('03) these variations in color occur in both sexes.

C. BREEDING HABITS

1. *Breeding season*

The following data indicate the beginning of the breeding season, as shown by the deposition of eggs, in northwestern Pennsylvania during a series of years:

1906	August 30	1909	August 29
1907	September 8	1910	September 1
1908	August 28	1911	September 4

The summer of 1907 was an unusually 'late season' as regards vegetation as well as the breeding season of Cryptobranchus. This indicates the probability that climatic conditions influence the time of spawning.

Egg-laying continues for a period of about two weeks. At the end of this time females have in a few instances been taken with the full complement of ripe eggs still in the ovary and showing signs of degeneration.

The occurrence of the breeding season of Cryptobranchus in the fall is in marked contrast to the habits of nearly all other urodeles. Some other urodeles which have a late breeding season are C. japonicus, which according to several authors (Sasaki, '87; Kerbert, '04; Ishikawa, '04; de Bussy '04 and '05) lays its eggs during the latter part of August and the early part of September; and Amphiuma, which according to McGregor ('99) breeds in midsummer. Among the anura, Scaphiopus holbrookii spawns during the summer, the time varying from June to August (Pike, '86; Hargitt, '88).

2. *External sexual characteristics*

The adult male may be recognized (Reese, '04) by the presence of a swollen ring about the cloaca, due to glands beneath the skin. This swelling is quite prominent during, and for a few weeks before the breeding season. I found it difficult to distinguish by external characteristics the sexes of a few specimens taken during the first week of July; during the latter part of July the males could easily be distinguished by the presence of the cloacal swelling. In a few males obtained and examined during the early part of November, the swelling was less pronounced than is usually the case during the breeding season. Females are characterized by the entire absence of the cloacal protuberance found in the male; also, the abdomen of the gravid female is slightly swollen.

3. *Sex ratio and sex segregation*

As a general rule, fewer females than males have been captured. The record of the sex of the great number of adults captured during the progress of the work is not complete, but the conclusion reached by later work is that the original ratio of 1:8 determined (Smith '07) during the fall of 1906 is much too large. In a series of years the proportion of females to males captured is about 1:2 or 1:3. These results are of course not conclusive as to the actual sex ratio; as will presently be explained, the sex ratio in the specimens captured varies for different times and places, and the true ratio may be disguised by the occurrence of seasonal segregation of the females from the more accessible localities.

In studying the distribution of the sexes throughout the year a distinction must be made between localities which experience has shown are chosen as breeding grounds, and other localities unsuited for breeding purposes. The breeding grounds are characterized by shallow water, a moderate current, and the presence of large flat rocks affording cover for cavities protected from the current. Elsewhere a swifter current, smaller rocks barely large enough to serve as cover, or deeper pools of quiet water, afford conditions in which Cryptobranchus can live, but which are not adapted for purposes of reproduction.

Studies of the sex ratio indicate a more or less perfect segregation of the sexes at certain seasons of the year. A dozen adults captured in June on the breeding grounds, by an assistant, proved to be all males. During the summer, search of the breeding grounds results in the capture of a few females and a much larger number of males; in localities unsuited for breeding one is more likely to find females, and males are seldom found in their immediate vicinity. Just before the breeding season one is more likely to find females on the breeding grounds, but the males are still considerably in excess, and there is apparently a tendency for the sexes to occur in groups: within a restricted area one may find only males, while within another area a short distance away one may find only females. At the height of the breeding season, both sexes are found on the breeding grounds in more nearly equal numbers.

For some days or weeks after the close of the breeding season the male remains in possession of the nest; females have never been found in nests containing eggs. At this time females have been found in considerable numbers in localities unsuitable for breeding, with no males in their immediate vicinity.

The general results of the studies of the sex ratio and the distribution of the sexes indicate that the males abound in localities suitable for breeding, throughout the year, and that they are less numerous elsewhere; it is positively established that the males alone are in possession of the nests after spawning takes place; and it is probable that there is a more or less perfect segregation of the females from the breeding grounds during a period extending from the close of the breeding season until the middle of the following summer.

In Necturus, segregation of the sexes at a certain season of the year seems to be more complete than is ever the case with Cryptobranchus allegheniensis. Eycleshymer ('06) says:

> In the autumn they are found in pairs or small groups. From this fact and others to be recorded later it is inferred that this is the mating season. . . . . During egg-laying [in the spring] the males are never found with the females, and where they remain is unknown.

4. The eggs

(*a*). *General history of the eggs and their envelopes before the time of spawning.* In an adult female of average size about 450 eggs are matured each season—225 from each ovary. In general the number is greater in the larger and presumably older females than in the smaller ones. At the approach of the breeding season the eggs which are about to become mature are readily distinguishable from the others by their much greater size and yolk content. The liberation of these eggs from the ovary and their passage down the oviduct takes place shortly before spawning. The exact date varies considerably in different individuals; for a week or ten days after the first cases of spawning, females may be found with mature eggs all still in place in the ovaries. The process of liberation of the eggs and their passage down the oviduct, once begun, must be accomplished with considerable rapidity; for out of more than a hundred females opened and examined during the breeding season in the course of several years, only four have been found in which the process was actually taking place. This state of affairs is in marked contrast to the condition in Bufo, where according to King ('05) the great majority of specimens collected soon after they had emerged from their hibernation contained eggs free in the body cavity. In three out of the four cases above mentioned for Cryptobranchus, eggs were found along the entire route: some still in place in the ovary; some free in the body cavity, for the most part collected at its anterior end, near the opening of the oviduct; others forming a procession down the oviduct; the remainder aggregated in the uterus. In the fourth case, the ripening eggs were found only in the body cavity, oviduct and uterus. The process takes place on the two sides of the body simultaneously.

During their passage down the oviduct the eggs receive their gelatinous outer envelopes, the product of the oviduct. At the upper end of the oviduct, the eggs collect in masses; a little further down, they are arranged in a solid row. In these parts of the oviduct the covering is absent or just beginning; the eggs are very soft, and elongated by pressure of the walls of the oviduct. In the middle and lower portions of the oviduct the eggs are distributed at fairly equal intervals; here the envelope is well formed, and consists of a capsule about each egg, and a slender connecting cord, giving a general resemblance to a string of beads.

After their descent through the oviduct, the eggs of each side of the body form a single string aggregated in a much twisted and tangled mass in the uterus. Considered as individuals without regard to their sequence in the string, the eggs display a striking regularity in their arrangment in the uterus, being packed in parallel spiral rows; but this is merely the result of mechanical pressure, as the string pursues a very sinuous and complicated course throughout the mass.

The egg capsules at the end of the uterus nearest the cloaca, hence those first formed, do not contain eggs; those nearest the oviduct, hence the last formed, are likewise devoid of eggs. These empty egg capsules are in general smaller than those that contain eggs, with a regular gradation in size from those at the extremity of the cord, which are scarcely more than a millimeter in diameter, up to those nearest the egg-containing capsules, where the diameter is only slightly less than normal. However small the size, these capsules are always perfectly formed, with a central spherical space; they are never solid. The 'empty' capsules contain a small amount of coelomic fluid in which are distinguishable under the microscope leucocytes, erythrocytes and yolk corpuscles. A cloudy mass of fluid with the same constituents occurs in the upper part of the uterus, outside of the egg envelopes. Similar capsules devoid of eggs are the 'wind eggs,' known in various vertebrates: birds, reptiles, sharks, chimaeroids.

As a result of experimental studies on the nature of the stimulus which causes the shell to be formed about the hen's egg, Pearl ('09) reached the following conclusions: (a) the stimulus which sets the shell-secreting glands of the fowl's oviduct into activity is mechanical rather than chemical in its nature; (b) the formation of a shell on the hen's egg is brought about by a strictly local reflex, and is not immediately dependent upon the activity of other portions of the reproductive system (nervous impulse or hormone formation). In this connection it is interesting to note that in Cryptobranchus the mechanical stimulus can hardly be the true cause of the formation of the capsule, since capsules are formed when only a small drop of coelomic fluid is present. Moreover it is here observed that coelomic fluid may pass down the oviduct without becoming enclosed in such capsules; on the other hand, every egg is provided with a capsule.

When distended with eggs, the uterus is spindle-shaped, about 10 cm. long, with a transverse diameter of about 4 cm. at its widest part. Its thin walls have a rich blood supply.

Apparently the eggs do not, as a rule, remain long in the uterus before spawning takes place. During the breeding season comparatively few females are found having eggs in the uteri; the majority of the females captured are either spent or with eggs still in place in the ovaries. Eggs taken from the uteri are, in the great majority of cases, capable of artificial fertilization; this subject will be more fully discussed later.

(*b*). *Oviposition, and nesting habits.* Under strictly natural conditions egg-laying takes place under cover of rocks in the bed of the stream; but in creek aquaria, arranged to afford conditions as natural as possible without too much cover, the process has been repeatedly observed.

Egg-laying begins slowly, a short string of eggs sometimes protruding from the cloaca for several hours before spawning begins in earnest. In the natural habitat, such short strings of eggs are often found in the open. Later, two long strings of eggs proceed slowly from the cloaca, one from each uterus; the majority of the eggs are then deposited more rapidly, in multiple strands, the process requiring less than five minutes. When egg-laying is completed, the strings are usually twisted together in a single tangled mass.

The 'nest' of Cryptobranchus allegheniensis has already been described as either a burrow or a natural cavity under a rock which is wholly or partially submerged. The eggs are not fastened in any way, but are protected by this sheltered position from being swept away by the current.

The nests of Cryptobranchus japonicus have been described by Ishikawa ('04), and closely resemble those of C. allegheniensis.

> Das Tier legte seine Eier in tiefe horizontal verlaufende Löcher, in denen das Wasser sehr ruhig ist. Manchmal is solch ein Loch 10 oder mehr Fuss tief und kaum für das Licht zugänglich. Die Brutstellen für die Eier sind aber nicht immer so tief. Oft fand ich Eier in einem Loch nicht tiefer als 3 oder 4 Fuss. Oeffnet man ein solches Loch, so findet man eine abgerundete Stelle, deren Boden ganz rein gehalten ist.

The nesting habits of Necturus have been described by Eycleshymer ('06), and the writer ('11). The eggs are attached singly by their gelatinous envelopes to the under side of a rock, board, or other object lying at the bottom of the water (figs. 55 and 56).

The eggs of Amphiuma found by Hay ('88 and '90) in an Arkansas swamp were in a comparatively dry situation, in a small excavation under a log several rods from the nearest water.

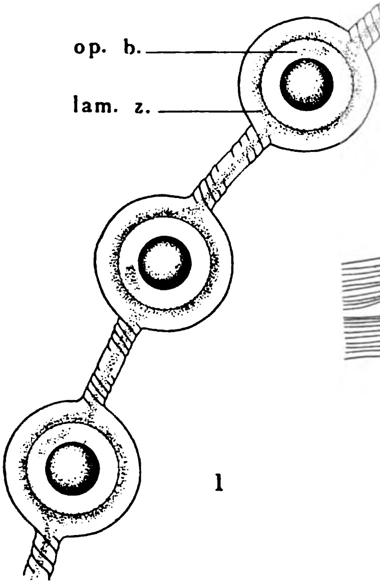
Brief reference to the nesting habits of some other amphibia has been made in previous papers (Smith, '06 and '07). Very remarkable are the nesting habits of the anuran Phyllomedusa, described by Budgett ('99); the eggs are deposited in a pocket made by bringing together the edges of a leaf overhanging the water.

Amongst the dipnoi, the nest of Protopterus (Budgett, '01 a and '01 b) is an oval hole filled with water and surrounded by swampy ground. The nest is at first entirely submerged, but by the partial drying up of the swamp it is left as an isolated pool. Lepidosiren (Kerr, '00) nests in a veritable burrow excavated in the black peaty soil of the swamp.

Nesting habits are well known in many teleosts, and in Amia (Dean, '96; Reighard '03). According to Budgett ('01 a) the crossopterygian Polypterus probably makes no nest, and certainly lays but few eggs at a time, these being scattered broadcast through the thick vegetation of the flooded grass lands. Comparison with Cryptobranchus suggests the possibility that these scattered eggs are but preliminary attempts at egg-laying.

(*c*). *The newly-laid egg and its envelopes.* (Figs. 54, 1 and 2.) In eggs taken from the uterus, the outer egg envelope or capsule fits closely about the egg proper; the envelopes are flaccid and much wrinkled. The capsule of the newly-laid egg takes up water rapidly; in the course of one or two hours a space, filled with fluid, appears between the egg and its capsule, sufficient to enable the egg to orient itself with the animal pole uppermost.

The egg proper is perfectly spherical when fresh, but it gradually becomes slightly oblate from the effects of gravity. It is about the size of a pea, and bright yellow in color—a rather deep yellow at the lower pole, grading to a very pale yellow at the upper. The general intensity of the yellow color varies considerably in eggs of different spawnings, but is quite uniform in eggs from the same female. The absence of black pigment is probably correlated with the fact that the eggs are laid in darkness: the



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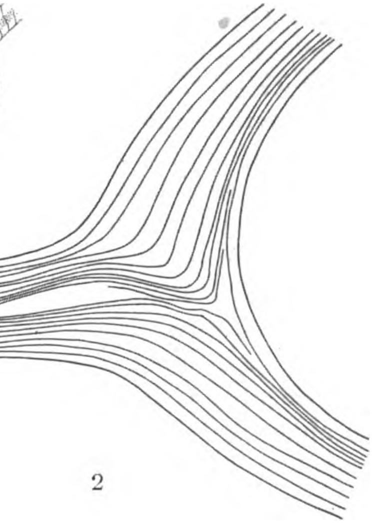

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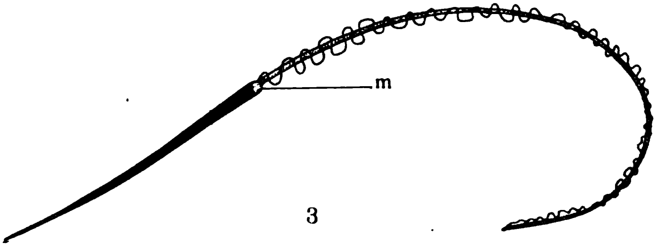

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Fig. 1 Eggs and egg envelopes of Cryptobranchus alleghemiensis, natural size. *op. b.*, opaque body; *lam. z.*, lamellar zone of envelope.

Fig. 2 Optical longitudinal section through the lamellar zone of the envelope in the region of junction of the egg capsule with the connecting cord. × 13.

Fig. 3 Spermatozoon. × 500. *m.*, middle piece.

eggs of Necturus, Plethodon, Spelerpes and Desmognathus, which are also laid under cover, are likewise unpigmented.

A very thin and transparent 'vitelline membrane'—the zona pellucida of the ovocyte—closely invests the egg; it is quite inconspicuous in fresh material. This is not the true cell wall of the egg, which, as described in detail on page 112 lies immediately within the vitelline membrane and represents in a modified form the zona radiata of the ovocyte.

Proceeding from within outward, the coverings of the egg may be enumerated as follows: (a) the cell wall; (b) the vitelline membrane lying in close contact with the preceding; and (c) the capsule or thick gelatinous outer envelope, which is separated from the vitelline membrane by a space filled with fluid.

During the first few hours after fertilization the capsule gradually becomes turgid by osmosis, becoming in this way a much more efficient protection to the egg; the space between the egg and its capsule is increased by the absorption of water and in this the egg almost floats, resting lightly on the lower inner surface of the capsule. When the eggs are removed from water the egg proper looks much larger than it really is, because magnified by the spherical capsule.

For a day or two the envelopes are quite soft and somewhat viscous, making it rather difficult to cut them with scissors in order to remove the eggs. Gradually the material of the envelopes becomes firmer. The connecting cord is at first quite elastic, but it loses this quality to a considerable extent after prolonged immersion in water.

Until after the eggs have been in water for several days, the outer layers of the envelopes are still cast into wavy folds or wrinkles, usually extending spirally about the capsules and the connecting cord. As a rule the spiral is constant in the direction in which it extends about the axis of the string in all portions of the cord and capsule. These spiral folds are usually most strongly marked at the ends of the cord adjacent to the capsule, and here they often persist (fig. 1), suggesting the chalazae of the hen's egg.

The envelope is perfectly transparent when fresh, except that wherever viewed tangentially its inner layers have a misty appearance, represented by the shaded zone in fig. 1, and due to a fine lamellar structure sketched in optical section in fig. 2. The misty appearance is caused by the diffusion of light passing through these concentric layers in a direction tangential to their surfaces. The core or axis of the connecting cord has the same misty appearance, due to a continuation of the lamellar structure. The various layers or lamellae of the gelatinous envelope are in intimate contact; there is no fluid-filled space between them such as occurs between the capsule and the vitelline membrane.

The inner layer of the lamellar core of the cord in some cases exhibits a marked twisted or spiral arrangement, like that of the inner portion of the cord connecting the eggs of Ichthyophis as described by the Sarasins ('87–'93).

The eggs of a given spawning are fairly uniform in size, but there is considerable variation in the size of eggs from different parents. The average dimensions of the living egg and its envelopes, after two days' immersion in water, are as follows:

Diameter of egg proper	6.2 mm.
Diameter of egg with envelope	18 mm.
Diameter of connecting cord	5 mm.
Distance of one egg from another, measured from center to center along the cord, about	30 mm.

A few egg capsules, particularly among the empty ones, are double, formed by the union of two capsules without a connecting cord. In such cases the cavities of the two capsules are usually separated only by a thin gelatinous septum; but all gradations occur between this condition and that in which two capsules are connected by an unusually short cord. Rarely, three capsules are closely approximated.

I have found a few instances in which two eggs occurred in the cavity of one simple capsule, without any separation by a gelatinous membrane. It would seem possible that double embryos might be formed in this way, by the fusion of the yolk masses of two such eggs; but this could not account for the only double embryo that I have found in nature, for in this case, to be described later, each embryo is half the normal size.

After fertilization, numerous spermatozoa are found imbedded in the egg capsule, and floating in the liquid between the capsule and the egg; they also occur in capsules that do not contain eggs. The spermatozoa occur singly, not in masses, and they are entirely absent from eggs taken from the uterus. Fertilization occurs only after the eggs have been deposited in the water (Smith, '07).

An envelope so tough and thick as that of Cryptobranchus must exert a decided selective power with regard to the spermatozoa; of a considerable number of spermatozoa simultaneously coming in contact with the envelope, the most vigorous, as well as the ones structurally best adapted, would succeed in first entering the egg.

Floating in the liquid between each egg and its envelope, there occurs a fairly large irregular and slightly opaque mass, in appearance like a faint white cloud (see fig. 1; this mass is also faintly shown in the photograph, fig. 54). Under the microscope it is found to consist of a clear viscous matrix in which are imbedded numerous leucocytes and occasionally a few erythrocytes. In fertilized eggs, this mass, which I have called ('07) the 'opaque body' sometimes contains spermatozoa, but they are not restricted to it, nor especially numerous in it. The opaque body is uniformly present in eggs that do not contain spermatozoa.

A mass similar in general appearance and location to that described above as the opaque body, is figured by Ishikawa ('04) within the egg capsule of Cryptobranchus japonicus. In the text he refers to these masses as 'Samenhaufen,' and speaks of the presence of spermatozoa within the egg capsules as evidence of internal fertilization. He considers it improbable that the spermatozoa are able to penetrate the egg capsule, and supposes that they are taken up into the oviduct before the egg capsules are formed.

DeBussy ('04, p. 11) found a mass ('vlokje') of similar appearance within the capsules of unfertilized eggs of C. japonicus. Under the microscope he found the mass to consist of a slimy substance containing red blood corpuscles and yolk granules, but no spermatozoa. He concludes that the presence of spermatozoa is not essential to the formation of the mass, but that they may merely form an element of it; hence that the name 'Samenhaufen' is scarcely justified. This conclusion is in essential agreement with my results on C. allegheniensis; it seems therefore that the masses called 'Samenhaufen' in C. japonicus by Ishikawa are of the same nature as the 'opaque bodies' of C. allegheniensis, and like them of no significance in fertilization. The opaque body apparently consists of coelomic fluid that has escaped into the oviduct.

The egg strings of Cryptobranchus japonicus as described by Ishikawa ('04) and deBussy ('04) closely resemble in structure those of C. allegheniensis. Both eggs and capsules of the Japanese form are slightly larger; according to Ishikawa the egg proper is about 7 mm. in diameter, and the capsule varies from 20 to 25 mm. in diameter in different spawnings.

The egg capsules of Amphiuma as described by Hay ('88 and '90) have the same general structure as those of Cryptobranchus. For an opportunity to examine one of Hay's specimens of the embryological material of Amphiuma, I am indebted to Prof. C. W. Hargitt, to whom the specimen had been presented by the finder. The egg capsule has a glistening surface like isinglass; it is thinner and apparently tougher, and the connecting cord more slender, than in Cryptobranchus. These peculiarities may be due in part to preservation in alcohol, which tends to produce the same condition in the envelopes of Cryptobranchus; but my impression is that the egg capsules of Amphiuma are better adapted to retain moisture when exposed to the air.

Other amphibians whose egg capsules are fastened together like a string of beads are Alytes, Ichthyophis, and Hypogeophis (Brauer '97).

The general appearance of the egg capsules of Necturus is shown in figs. 55 and 56; some further details of structure are shown in fig. 4. There are three layers to the gelatinous envelope: (a) a comparatively thin but very dense inner layer, consisting of several lamellae; (b) a thicker median layer of moderate density, consisting of many lamellae; and (c) a very thick outer layer of homogeneous material, much less dense than either of the preceding. This outer layer is produced to form the stalk by which the capsule is attached to some solid object. As seen in optical section, the lamellae of the two inner layers have a somewhat sinuous or wavy outline. Leaving the stalk out of account, the entire structure bears a close resemblance to the gelatinous envelopes of the frog's egg. In the early stages of development of the embryo, the dense inner layer of the capsule fits so closely that it is not clearly differentiated from the embryo; this layer is best studied after the embryo has passed the gastrula stage, when a narrow space, filled with liquid, appears between the embryo and its capsule (see fig. 4). This space is not strictly homologous with the similar space that appears much earlier in the egg of Cryptobranchus; for in Cryptobranchus the space appears between the gelatinous envelope and the vitelline membrane (zona pellucida of the ovarian egg) which remains in close contact with the egg, while in Necturus the vitelline membrane apparently func-

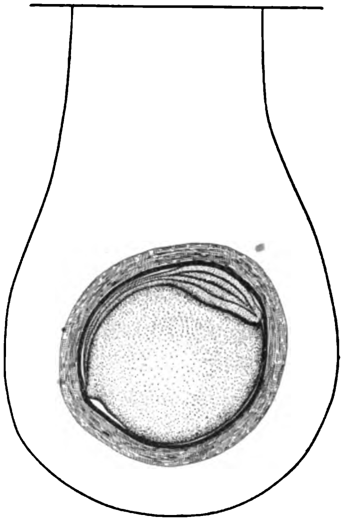


Fig. 4 Optical section through an egg capsule, and surface view of an embryo, of Necturus. The embryo is shown in a stage with neural folds, when the capsule is slightly separated from it by a space filled with water. × 4.

tions as the innermost lamella of the capsule. The entire inner layer of the capsule of Necturus has a tough consistency similar to that of the vitelline membrane of Cryptobranchus; this perhaps is the reason why it is so slow in enlarging.

In Necturus the egg proper is slightly smaller than that of Cryptobranchus; in the early cleavage stages it measures about 5.8 mm. in diameter.

A general description of the gelatinous envelopes of several species of Amblystoma has been given in a previous paper (Smith '11).

5. *The sperm*

The spermatozoon (fig. 3) has been figured by Reese ('04), who fails, however, to picture the middle-piece. The spermatozoon is about 225μ long, and stout in structure as compared with the spermatozoa of Amblystoma and Diemyctylus. The head, excepting the acrosome, stains deeply with Delafield's haematoxylin. The acrosome appears to be uniformly tapering, not spear-shaped as in Amphiuma (described by McGregor, '99). As stated by McGregor ('99) the middle-piece in Cryptobranchus is very short in comparison with that of other urodeles. The tail-piece is provided with an undulating membrane, bordered with a convoluted filament.

The ripe spermatozoon is motile, as regards both shaft and filament; but the spermatozoon as a whole is not so flexible as the more slender spermatozoa of Amblystoma and Diemyctylus. This greater rigidity of the spermatozoon is perhaps correlated with the method of fertilization: the spermatozoon must penetrate the tough and thick egg capsule after a brief exposure of the latter to the hardening effects of water.

In seminal fluid obtained from occasional individuals, an oval mass of granular protoplasm, about 13μ by 16μ, surrounds the posterior part of the head of the spermatozoon. The long axis of this bead-like mass coincides with that of the spermatozoon. I have found a similar mass of protoplasm present in spermatozoa from some individuals of Amblystoma punctatum, but here the oval mass usually occurs about the junction of the head and middle-piece. Probably the condition observed in the two species represents a late developmental stage of the spermatozoon—the metamorphosis of the spermatid into the spermatozoon is not quite complete.

The amount of seminal fluid present at one time in the vasa deferentia of a ripe male is very great in proportion to the size of the animal—a condition correlated, doubtless, with external fertilization. In one instance 20 cc. of seminal fluid was readily stripped from a single male.

6. The method of fertilization

The method of fertilization (external) has already been described (Smith, '07); subsequent observations have supplemented this account only in the fact, discussed later, that a single male may spawn with more than one female.

The method of external fertilization is well adapted to the normal breeding conditions. The 'nest' of Cryptobranchus consists of a hollow under rocks, a confined space protected from the current, and filled with very quiet water. As has been shown, the amount of milt that may be discharged at one time by a single male is considerable; in the case of a pair that spawned while being carried in a pail of water, it was sufficient to turn several quarts of water milky white. Such a quantity of sperm set free in the confined space of the nest would become diffused, especially when stirred about by the movements of the animals, so that every egg would be quickly reached and fertilized. As a matter of fact, few unfertilized eggs are found.

So far as I have been able to learn, this is the only case of external fertilization recorded for the urodeles. The inconclusive observations of Kerbert ('04) on Cryptobranchus japonicus, and Kunitomo ('10) on Hynobius, suggests to me that external fertilization may take place in these forms.

In Necturus, from the observations of Kingsbury ('95) it seems certain that internal fertilization takes place. A compound receptaculum seminis or spermatheca is present in the female; spermatozoa have been found in these spermathecae during the fall and winter, which suggests an autumnal fertilization, though it is possible that spermatozoa are left over from a spring fertilization. The method of transference of the seminal fluid from the male to the seminal receptacle of the female is unknown.

The breeding habits of some urodeles in which internal fertilization takes place by means of spermatophores (e. g., Amblystoma punctatum and Diemyctylus) have been considered by the writer in former papers (Smith, '07, '10 and '11). Fertilization is external in the anura, internal in the apoda.

In the elasmobranchs and holocephali, fertilization is internal. In the crossopterygian Polypterus (Harrington, '99; Kerr, '07 b), during the breeding season the anal fin of the male is modified in such a manner as to suggest internal fertilization; or possibly it serves to direct the sperm against the stream of eggs issuing from the female. Nothing conclusive is known regarding the method of fertilization in the dipnoi. In teleostean fishes, with a few exceptions, fertilization is external; e.g., as in Chrosomus (Smith, '08).

The question whether external fertilization in Cryptobranchus is primitive or secondarily acquired will be discussed under phylogenetic considerations in a later section.

7. *The brooding habit*

In a previous paper (Smith, '07) a paternal brooding habit was described for Cryptobranchus. This was observed in aquaria, and more extensively under natural conditions.

The data on the existence of a paternal brooding habit under natural conditions, while necessarily incomplete, are quite conclusive. In one case, a male occupying a nest containing eggs was observed to fight and drive away several males and a spent female which were attempting to enter the nest (Smith, '07); in another case, a male occupying a nest containing eggs was observed to oppose the attempt of a single male to enter the nest. It is not always possible to tell whether an adult is present in the nest; the rock may be too large to overturn, and while the eggs may be obtained by tilting the rock with a crow-bar, this method is not always successful in dislodging the adult. In cases where the rock is lifted and overturned, the water is discolored and the hellbender, aided by its protective coloration and the swift current, may escape. When seen, however, it may usually be captured. A record kept for six years ('06–'11) shows that from twenty-nine nests containing eggs a male was captured in ten cases, a female never.

The duration of the brooding habit has not been definitely determined, and perhaps varies greatly. In different nests in which a male was present, eggs were found in various stages of development up to about three weeks old; unfortunately I was obliged to discontinue field work at a date varying from two to four weeks after the beginning of the breeding season. In no case where the eggs were in an advanced stage of development can it be recorded that the male had been continuously present, or even that he was the same male that fertilized the eggs; but the entire absence of females from nests containing eggs is significant.

With regard to the origin of this paternal brooding instinct two suggestions (Smith, '07) were made: (*a*) the brooding instinct may have originated in connection with the feeding habit; or (*b*) in holding the nest the male may be primarily concerned in awaiting the coming of another ripe female. Both views assumed that in Cryptobranchus we have an example of the brooding habit in an incipient state. Further observations indicate that the brooding habit is well established and manifested as a distinct impulse from the moment of fertilization; its origin is thus thrown back into the remote past, and concerning it we can only speculate.

The evidence for the first interpretation may be briefly stated as follows: Both sexes are voracious eaters of the newly-laid eggs; during the spawning season the majority of the adults taken have the stomach filled with eggs. There is evidence that the females, when opportunity is afforded, gorge themselves with eggs more freely than the males. The number of eggs found in the stomach of a single adult usually ranges from fifteen to twenty-five, a number sometimes greatly exceeded in the stomachs of spent females. In one case, in which the body of a spent female appeared greatly swollen, the stomach was found to be greatly distended with eggs. When removed and measured by displacement of water, the stomach and its contents were found to have a bulk of over 200 cc. The mouth also was full of eggs, and strings of eggs protruded from the pharyngeal openings. The quantity of eggs present seemed to represent almost an entire spawning. The feat of swallowing such a quantity of eggs would seem possible only if they were taken before the swelling of their envelopes.

The digestive processes of the hellbender are extremely slow, and I have taken undigested eggs from the stomach a week after they were eaten. Under these conditions the presence of a single male hellbender in the nest is in the main protective. On account of the small number of eggs eaten at once, and the slowness of his digestive processes, fewer eggs are eaten than would be the case if other hellbenders, and especially the spent females, had free access to the nest.

As previously noted, a male has been observed to fight and drive away a spent female and several males that were attempting to enter the nest. The male in such cases has the advantage over the female because of the weakened condition of the latter; as regards the other males, he has the advantage of position.

The facts suggest that the male, in thus driving away others of his own kind, may be primarily concerned in guarding his own food supply; this guarding habit may become modified into a true brooding instinct. But it is difficult to believe that the male, after having filled his stomach with eggs, would any longer be concerned with the fate of the remaining eggs on account of their value as food.

According to the second interpretation, the male may hold the nest in expectation of the coming of another ripe female; an extension of this habit may give rise to the brooding instinct. According to this view, the brooding instinct has its origin in the breeding habit.

The reception of a ripe female by a male guarding eggs has not been directly observed, but the following data afford sufficient evidence on this subject: Out of twenty-nine nests examined during the seasons of 1906–1911 inclusive, eleven were found to contain eggs of at least two different spawnings, the product of different females. The eggs were in different stages of development, hence fertilized at different times. In two cases the number of eggs present in a single nest was sufficient to represent at least three spawnings by different females. It is possible to determine this with considerable certainty, for the number of eggs matured by a single female each season is limited, and these are all laid at one time. Moreover it is known that the intensity of the yellow color of the eggs is constant for all the eggs of a single female, but varies considerably in eggs from different individuals. In view of the observed vigilance and effectiveness of the male in possession of the nest in driving away other males, it is highly improbable that successive pairs of adults have occupied the nest; hence the facts indicate that the same male has spawned with successive females.

The second hypothesis seems supported by better evidence than the first; but while it is entirely possible that such may have been the origin of the habit in the remote past, there is evidence that at present the eggs are the object of paternal care from the time of fertilization, and this brooding instinct is only temporarily overcome by hunger or diverted by the breeding instinct. The behavior of males breeding in aquaria strongly suggests this: after fertilizing the eggs the male usually remains close beside them or crawls under or amongst them.

Concerning the brooding behavior of some specimens of Cryptobranchus japonicus in captivity Kerbert ('04) says:

> Nach Beendigung der Eiablage legte sich das Weibchen offenbar in grösster Ermattung in eine Ecke des Behälters hin und kümmerte sich um das Gelege gar nicht mehr. Das Männchen hingegen hat seitdem die Eiermasse nicht verlassen—ja sogar *die Brut fortwährend bewacht.* . . . . . Denn sobald das Weibchen die Eiermasse zu nahe kam, stürzte das Männchen in sichtbarer Wut auf die Mutter los und vertrieb sie. . . . . . kriecht der Männliche Riesensalamander zwischen den verschiedenen Strangen der Eiermasse hindurch und bleibt dan von der Eiermasse umhüllt liegen, oder er legt sich einfach neben die Eiermasse hin. In beiden Fällen aber hält er, hauptsächlich durch eine pendelartige Bewegung des ganzen Körpers, von Zeit zu Zeit die ganze Eiermasse in Bewegung. Durch diese Bewegung entsteht eine für den Atmungsprozess der Eier und Embryonen höchst wichtige Wasserströmung, wahrend die Lage der Eiermasse hierdurch gleichzeitig fortwährend wechselt.

It thus appears that the paternal brooding instinct in both species of Cryptobranchus is manifested from the moment the eggs are fertilized, though in C. allegheniensis at least it may be temporarily inhibited or overcome by hunger or by the breeding instinct. The brooding habit of Cryptobranchus is undoubtedly very old, and we must look to other forms to find examples of it in the incipient condition.

According to Whitman ('98) there are three distinct elements in brooding behavior: (*a*) the disposition to remain with or over the eggs; (*b*) the disposition to resist and to drive away enemies; and (*c*) periodicity. The first of these elements has its origin in the need for rest, protection to the offspring being at first incidental. The second element, pugnacity, is periodical and a part of the reproductive cycle. The third element, periodicity, is apparently an attribute of the two other elements, based on physiological conditions; its adaptiveness lies in correlating the other two elements with the hatching period of the eggs.

In Cryptobranchus, after spawning, the female is evidently much the weaker of the two; as a matter of observed fact, she is driven away by the stronger and more pugnacious male. It can scarcely be the need for rest that keeps the male in the nest, since he maintains exclusive possession at the cost of alert watchfulness and occasional combat. If the element of weakness were the important factor in initiating the brooding habit, we should expect the female rather than the male to remain in the nest. It may be that primitively the brooding impulse is a phase of the reproductive cycle that applies to both sexes, the female losing it on account of her hungry and exhausted condition due to the accumulation of a large amount of yolk in the egg. Perhaps (for this suggestion I am indebted to Professor S. J. Holmes) on the part of the male there is involved a proprietary interest in the nest, which he has chosen and in part excavated, and which he occupies as an advantageous breeding place and as a more or less permanent home.

To obtain conclusive evidence regarding the origin of the brooding habit one must study a series of closely related forms illustrating the habit in the making. In Cryptobranchus it appears that the habit is well established; it is improbable that the question can be settled by the study of this form alone, and the data here given are presented only in the hope that they may contribute something toward the final solution of the problem.

Concerning the brooding habit of C. japonicus in its natural habitat Ishikawa ('04) says: "Fast in jedem Loch, wo man von Ende August bis zu Anfang October ein weibliches Tier gefunden hat, findet man einen Eiklumpen. Dieser Umstand lässt schon vermuthen, dass das Tier eine Brutpflege hat wie Ichthyophis oder wie so viele andere Amphibien." Kerbert, however, asserts ('04) that it is the male that guards the eggs, and states that the sex of his specimens was carefully determined.

Other amphibia known to possess brooding habits are the urodeles Desmognathus and Plethodon; the caecilians Ichthyophis and Hypogeophis; Alytes and several other anura (Wiedersheim '00). In the cases of Desmognathus, Plethodon, Ichthyophis and Hypogeophis the female is said to care for the eggs; in the case of Alytes, the male.

The brooding habit seems to be lacking in Necturus. According to Eycleshymer ('06), Necturus sometimes eats the eggs of its own species.

The brooding habit is well known in many teleosts, and in Amia (Dean, '96; Reighard, '03); it is well developed in the lungfishes Protopterus (Budgett, '01 a and '01 b), and Lepidosiren (Kerr, '00). I can find no record of any observations pointing to the existence of a brooding habit in the crossopterygii.

D. SUMMARY

The breeding season of Cryptobranchus allegheniensis in northwestern Pennsylvania begins about the first of September and lasts about two weeks.

There is a tendency toward segregation of the females from the breeding grounds during a period extending from the close of the breeding season until the middle of the following summer.

About 450 eggs are matured each year by an adult female of average size. The egg capsules from each oviduct are fastened together in a single string. At the ends of each string are formed some small but perfect capsules which do not contain eggs.

The eggs are unpigmented, heavily yolk-laden and strongly telolecithal.

The nest consists of a submerged cavity under a rock in the bed of the stream. The cavity is sometimes in part the work of the animal.

Fertilization is external.

There is a paternal brooding habit, which is manifested from the moment of fertilization. The origin of this habit is problematical.

III. METHODS AND TECHNIQUE

A. COLLECTION AND CARE OF LIVING MATERIAL

To insure a convenient supply of adults for various purposes, these were collected before and during the breeding season and placed in a large creek aquarium, constructed of wire netting and placed in shallow water with a gentle current. This arrangement of the aquarium afforded abundant aëration; flat stones placed on the bottom provided cover; in general the conditions closely resembled those of the natural environment. The aquarium proved of great value as a means of insuring a supply of adults for use at frequent intervals in securing material for the study of ovogenesis, maturation, fertilization and the early cleavage stages.

Artificial fertilization was often resorted to in order to control the time of fertilization for the study of fertilization and early cleavage stages, and occasionally eggs were used that had been deposited and fertilized by specimens in captivity; but the greater part of the material used for the study of the development was obtained from the nests of the animals in their natural environment.

At first the problem of keeping the eggs alive in a favorable environment while studying their development promised some difficulty. Early attempts to keep the eggs in creek aquaria met with disastrous failure through the attacks of water-mould. The method finally employed was to keep the eggs in shallow earthenware dishes containing well water, in a cool cellar; a limited number of eggs were placed in each dish, and the water changed daily. Under these conditions they developed normally. During the early autumn all the laboratory work on the living egg, and the preservation of material, were carried on in this cellar, so that at no time were the eggs subjected to an unfavorable temperature. The eggs were in general shielded from the light; but for working purposes both direct and diffused sunlight, or a Welsbach light, were used.

On account of teaching duties observations in the field have never extended quite to the time of hatching, consequently it has been necessary to transport the living embryo for considerable distances. In the case of embryos taken after the closure of the neural folds, material shipped in cool weather by express, in a pail containing shallow water, did quite as well as material which was given personal care during transportation and for which the temperature was regulated with ice; in both cases the embryos developed normally. Younger embryos require much greater care in transportation; material in cleavage and gastrula stages shipped by express has usually died or developed abnormally, perhaps in the main because of untimely warm weather; all such material was discarded. Material kept in the laboratory thrives in shallow dishes containing well water, the dishes being partly immersed in cool running water; no artificial aëration is necessary. As a check on possible abnormalities in material that has been transported, I have had a series of late stages preserved from material kept without transportation.

B. FIXATION AND PRESERVATION OF MATERIAL

The envelopes may be removed in any stage without much difficulty, by means of scissors. This is very easily done after the eggs have been in water for several days, since the envelopes become inflated. For earlier stages, more care is necessary. Eggs from the uterus, and fertilization stages, may be handled more rapidly by fixing in Solution B (see below) before the removal of the envelopes; they may be preserved thus in formalin, but not in alcohol. After fixation the envelopes become brittle and may readily be removed with needles. Comparison with eggs fixed after the removal of the envelopes shows no essential difference in the results.

The fixation of such large and heavily yolk-laden holoblastic eggs presented a problem of considerable difficulty. A great variety of the usual fixing fluids were tried, but none of them succeeded without modification. After extensive experimentation, the mixture described below as Solution B was found to be very satisfactory for all the yolk-laden stages, for surface study, photography and for sectioning.

The following fixing solutions were found useful for the purposes indicated:

Solution A. Formalin, 10 per cent. Useful for preserving eggs in the envelopes for demonstration purposes, or for the study of the envelopes, as it leaves the envelopes clear and preserves the eggs in their natural color. Formalin is of some value for the surface study of cleavage, as it brings out the faint cleavage furrows of the lower hemisphere with great distinctness, and occasionally gives remarkably good preparations for the surface study of the cleavage of the upper hemisphere. In general the fixation of the micromeres is unsatisfactory, both for surface study and for sectioning. Formalin is unsurpassed for fixing larvae for museum purposes; for permanent preservation they should be changed to alcohol.

Solution B. Bichromate-acetic-formalin. The following proportions must be quite strictly adhered to:

Potassium bichromate	1	gram
Glacial acetic acid	2¼	cc.
Schering's formalin, added at the time of using	5	cc.
Water	92	cc.

Fix about forty-eight hours in plenty of the solution, at a low temperature; change the solution once or twice.

Rinse in water and wash in 5 per cent formalin, in the dark, for at least two weeks, changing the formalin as often as it becomes discolored; preserve in 5 per cent formalin. Preservation in alcohol also gives good results for sectioning, but is not so good for surface study nor for photography.

During the process of washing in formalin the color changes from yellow to green. The yolk becomes dark green, while the blastodisc or embryo proper is much lighter in color, giving a sharp differentiation of the protoplasmic portions of the egg. The form of the egg is preserved perfectly, and remarkably good definition for surface study is secured. The eggs are easily sectioned by the paraffin method.

Not until after the closure of the neural folds is it possible to alter the proportions in the formula as given above without injury to the form of the embryo; an increase in the proportion of potassium bichromate results in the collapse of the embryo when in melted paraffin if not in an earlier stage of the process of preparation for imbedding. For later stages the proportion of potassium bichromate may be slightly increased (e.g., to 1½ per cent), without detriment to the surface features and perhaps with some gain in the histological results.

Solution C. Sublimate-acetic-formalin.

Saturated solution corrosive sublimate in 10 per cent formalin	97½ parts
Glacial acetic acid	2½ parts

Fix for a few hours, then transfer to formalin for a few days to insure thorough fixation of the yolk. Wash and preserve in either formalin or alcohol.

This is not so satisfactory a fixing solution as Solution B, but may be used for comparison. For surface study the results, especially in the early stages, are decidedly inferior to those secured with Solution B. For sectioning, good results are secured in the early cleavage stages and after the closure of the neural folds; the mercury crystals must be removed by prolonged treatment with iodin. In the blastula and gastrula stages the embryo usually collapses during the process of preparing for imbedding.

Solution D: Lavdowsky's.

Formalin	10 parts
Alcohol, 95 per cent	50 parts
Glacial acetic acid	2 parts
Water	40 parts

Fix for several days; preserve in 70 per cent or 80 per cent alcohol.

This mixture is especially useful for the yolk-laden ovarian eggs, and for maturation stages; it is not very satisfactory for embryonic stages. Envelopes, if present, must be removed before the eggs are fixed in this solution. The best results are obtained by sectioning the material soon after preservation.

Solution E: Zenker's. This mixture was found most useful for the early stages of ovogenesis, before the formation of any considerable amount of yolk. It is not good for embryonic stages, unless parts of the embryo are to be dissected off from the yolk before sectioning. It gives very inferior preparations for surface study in every stage.

For the early stages of ovogenesis, before the formation of yolk, both Flemming's and Bouin's solutions were used with fair results. For larvae after the disappearance of the yolk sac, Tellyesnicky's, Zenker's, or almost any good fixing solution may be used.

Of the various mixtures experimented with for the yolk-laden stages, those containing picric acid proved to be the very worst. The invariable result of the use of a solution containing picric acid was to cause the egg to disintegrate.

In preserving the embryological material of Necturus, Solution B was principally used. In the early stages of development, before the formation of the neural folds, the results are not so uniformly good as with Cryptobranchus; this is perhaps due to the fact that in these stages the eggs of Necturus are almost necessarily preserved before the removal of the very closely-fitting gelatinous envelopes. In successfully preserved eggs in the cleavage stages, the furrows of the upper hemisphere are more conspicuous and the contour of the micromeres more rounded, than in Cryptobranchus; they are thus, except for difficulties arising from the character of the envelopes, more favorable objects for photography. Late gastrula and neural groove stages of Necturus, preserved by this method, are rarely so favorable for surface study as the same stages in Cryptobranchus. After the formation of the neural folds, when a space has appeared between the envelope and the egg, the embryos of Necturus are preserved with very uniform success, whether fixed before or after the removal of their envelopes. In particular, stages after the closure of the neural folds give a sharpness of detail in the surface features rarely found in Cryptobranchus; these stages of Necturus are very favorable objects for photography.

C. SECTIONING AND STAINING

Kerr ('01), in describing the technique employed in studying the egg of Lepidosiren, has well said: "The investigation of a holoblastic egg 7 mm. in diameter and packed with yolk involves great technical difficulties, for the whole of each egg has to be converted into thin sections. The full extent of these difficulties will only be appreciated by embryologists who have essayed a similar task." In sectioning the heavily yolk-laden stages, Kerr used the celloidin method, and a combination of the celloidin and paraffin methods. DeBussy ('04) used the celloidin method in studying the cleavage stages of Cryptobranchus japonicus.

For sectioning the embryological material of Cryptobranchus allegheniensis and Necturus I have used the paraffin method exclusively; success with this method was found to be entirely a matter of careful attention to technique. The most important considerations are proper fixation and washing, and thorough infiltration with paraffin. In handling serial sections of large numbers of these eggs the advantage of the paraffin method is obvious.

With regard to staining, for general purposes the best results were obtained by staining *in toto* with Grenacher's borax carmine, and counterstaining on the slide with Lyons blue in absolute alcohol; to the Lyons blue solution sufficient picric acid was added to turn it green. By this method the effect of a triple stain, with excellent differentiation, is obtained. The chromatin is stained red, cell walls and cytoplasm blue; the yolk is first stained red by the borax carmine, but turns green in the counterstain. It is usually best to cut short the action of the counterstain at a time when the smaller yolk particles are stained green, while the larger ones are left red. The method has the advantage of rapidity, an important consideration when great series of large sections are to be handled in considerable numbers.

In sectioning and staining the early cleavage stages the exact mode of procedure is as follows:

From formalin pass the eggs to alcohol, 35 per cent, 50 per cent, two hours each.

Grenacher's borax carmine in 70 per cent alcohol, about two days.

Acid alcohol (0.25 per cent HCl in 70 per cent alcohol), about two hours.

Ninety-five per cent alcohol, two to twelve hours; 100 per cent alcohol, two to three hours.

Xylol, four to ten hours.

Paraffin with melting point 52° C. (at a temperature not exceeding 55° C.), two days. Change the paraffin at least once.

Imbed in a paper box, hardening the block under alcohol.

Cut sections 10μ to 15μ thick, using a Minot rotary microtome.

Counterstain on the slide with Lyons blue and picric acid mixture in absolute alcohol.

Wash in xylol long enough to destain slightly.

Mount in Canada balsam.

Early stages require longer for fluids (especially paraffin) to penetrate than do later stages. For the yolk-laden ovarian eggs, and maturation and fertilization stages, from two to three days in borax carmine, and about three days in melted paraffin, are necessary. I have found no serious ill effects in these stages from this prolonged immersion in paraffin at the temperature given.

Material fixed in Lavdowsky's solution stains and infiltrates more rapidly than with the other methods of fixation; also the yolk is less likely to crumble.

Late cleavage, and gastrula stages, are penetrated by the various fluids more rapidly than the early cleavage stages, so that the time may be reduced to two-thirds or one half. For still later stages, there is a further gradual reduction in the length of time required.

During the present year, at the suggestion of Professor Wilson, I have employed a slight modification of the method described above. After being cleared in xylol, the objects were left several days in a mixture of xylol and paraffin at about 38° C. By this preliminary treatment, the time required for infiltration with melted paraffin at a high temperature was reduced at least one-half, with some improvement in the quality of the preparations.

IV. THE EXTERNAL HISTORY OF THE EGG BEFORE CLEAVAGE

Some superficial aspects of the history of the egg before cleavage have already been considered in connection with the account of the breeding habits.

A. EXTERNAL CHANGES PRECEDING AND ACCOMPANYING MATURATION

Except where otherwise mentioned, the observations recorded under this heading were made on the living egg.

If the ovary of an adult Cryptobranchus be examined at any time during the summer, the eggs which are about to become mature are readily distinguishable by their much greater size and yolk content.

In the living ovaries of adults taken about the middle of August, the eggs show no positive surface indications of a telolecithal structure. The same eggs fixed by a variety of methods show a circular area or 'calotte' about 60° in diameter, which is somewhat lighter in color than the remaining surface of the egg. On account of its large size the egg now causes the ovarian wall to bulge strongly outward. In general the pale circular area is situated in the center of the more exposed hemisphere of the egg, and is not so profusely covered with ovarian blood-vessels as the remainder of this hemisphere, but this relation is not always exact.

Sections show that the calotte is the outward expression of a peripheral disc-shaped region richer in protoplasm and small yolk granules than the remainder of the egg; in the center of this disc lies the germinal vesicle. From its homologue in the teleostean egg I shall call this region the *germinal disc* or *blastodisc;* in surface views it may be referred to by the same names, or more strictly speaking, as the *germinal area.* Fixation serves to accentuate the optical differences between the germinal disc and the remainder of the egg, making the germinal area visible in preserved material at an earlier stage than in the living egg. The center of the germinal area defines the animal pole of the egg.

Shortly before the egg is ready to leave the ovary, the germinal vesicle appears at the very surface, at the center of the germinal area which is now visible in the living egg. After remaining here for a length of time that has not been accurately determined, the germinal vesicle disappears from view leaving only a faint dark spot to mark its former site.

In most ovaries obtained about the time of the beginning of the breeding season (the last week in August and the first week in September), all stages in the emergence of the germinal vesicle will be found; in some eggs the germinal vesicle has not yet reached the surface, but in a considerable proportion of cases it will be found exposed in varying degrees (see fig. 53).

The phenomena concerned with the appearance of the germinal vesicle at the surface are very striking, owing to the large size of the germinal vesicle, the sharp contrast between its transparent fluid contents and the surrounding opaque substance of the egg, and the distinct appearance of several opaque-white bodies, presumably nucleoli, within the germinal vesicle. All this may be seen even with the naked eye.

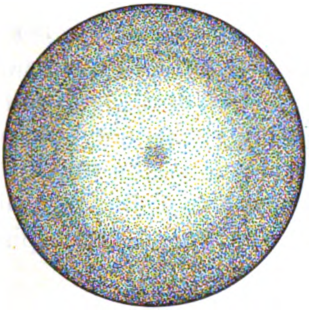
In order to obtain the sequence of the changes occurring in a single egg during this stage, many individual eggs were isolated in normal salt solution, or identified while in position in the ovary, and kept under observation for several hours. In the case of the first ovary studied during the fall of 1907, some of these eggs changed sufficiently before death ensued, to enable me, by combining several individual histories, to get a fairly complete idea of the normal course of events in a given egg. But in succeeding years, although several dozen ovaries containing eggs in this stage have been studied in females recently killed or anaesthetized with chloretone, no marked changes could be detected. Hence in the following account dependence is placed chiefly on a comparison of individual eggs in the same freshly-exposed ovary, and a comparison of ovaries in slightly different stages of development. In particular, incipient stages in the approach of the germinal vesicle to the surface could be distinguished from possible later stages in which it has disappeared from view, through a comparison of ovaries such as those described above, with others in which nearly all the eggs had been set free from the ovary.

The first indication of the approach of the germinal vesicle to the surface is the appearance of a faint dark spot, 1 to 2 mm. in diameter, in the center of the blastodisc. This dark spot grows more distinct; it is the optical effect of the scarcely-submerged germinal vesicle. Within this large dark area appears a small sharply defined much darker area, circular in outline, which grows at the expense of the larger and fainter dark area. At the time of its first appearance the small dark area is sometimes seen to pulsate slowly, quiver and change form, disappear and reappear. This small dark spot is a portion of the germinal vesicle which is actually in contact with the zona radiata (see section V). It may increase in size until almost an entire hemisphere of the germinal vesicle is exposed. In light of moderate intensity the germinal vesicle appears as a deep, dark well of transparent substance walled in by the opaque material of the blastodisc; in strong sunlight one may see within the germinal vesicle the reflection of the bright yellow yolk beneath. Several opaque-white bodies of various sizes appear within the germinal vesicle; these are probably nucleoli, though the largest ones are much larger than the nucleoli shown in sections.

An ovarian egg dissected out and immersed in water at the time of the appearance of the germinal vesicle at the surface orients itself with the animal pole upward.

The actual disappearance of the germinal vesicle from the surface, and the relation of this process to the rupture of the nuclear wall, have not been satisfactorily observed. Whether the germinal vesicle recedes slightly from the surface before or during the rupture of its wall, or disintegrates at the very surface, has not been positively established; it is possible that all three conditions occur in different eggs. In several cases there seemed to be a welling-up of material from the germinal vesicle which spread out to form a broad crater at the surface; in other cases the appearances favored the impression of a slight subsidence of the germinal vesicle. It is possible that the egg has never been observed at the exact time of the rupture of the nuclear wall, for though a large number of eggs from ovaries containing eggs with the germinal vesicle at the surface have been sectioned, in none of these eggs has the nuclear wall been found ruptured.

Several females have been taken in which only a few eggs remained in the ovary, the others being found in the body cavity, oviduct and uterus. The ovarian eggs of such specimens were invariably found to be in a later stage than those just described: sections showed that the dissolution of the germinal vesicle was complete, and in surface views these eggs showed a small faint dark spot or slight depression at the animal pole (see fig. 5).



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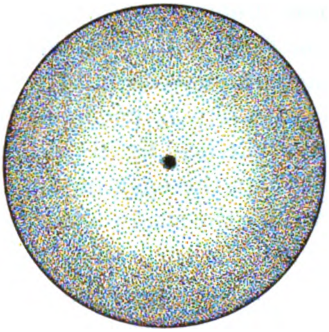

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Fig. 5 Surface view of the animal hemisphere of an egg of Cryptobranchus allegheniensis ready to leave the ovary, after the rupture of the germinal vesicle. The lightly stippled area indicates the blastodisc. Sketched from the living egg. × 7.

Fig. 6 Surface view of the animal hemisphere of an egg taken from the uterus, ready for fertilization, showing pit at the center of the blastodisc. Sketched from preserved material. × 7.

The dark spot is sometimes surrounded by a tumid ring, but this condition is probably pathological.

At the time of the escape of the egg from the ovary and its passage through the body cavity and upper oviduct, the egg seems softer in consistency than at other times. Some fixing solutions, particularly Lavdowsky's, which usually preserve perfectly the spherical form of the egg, now fix it as an irregularly-shaped mass. This plasticity may be of use to the egg in its escape from the ovary and passage down the oviduct.

In eggs taken from the lower oviduct there is found a slight extension of the blastodisc and a marked increase in the intensity of its differentiation, both in living and preserved material. Moreover, outside the rather indefinite limits of the blastodisc proper there seems to be a continuation of the same sort ot material as an extremely thin whitish superficial layer extending beyond the equator and well into the lower hemisphere.

In eggs entering the uterus the blastodisc is well differentiated throughout an area about 90° in diameter, while the entire remaining surface of the egg shows a slight paleness as compared with earlier stages.

The dark spot or shallow depression at the animal pole persists, though often very faintly, up to about the time of fertilization, when its site is occupied by a minute but deep and sharply-defined pit (see fig. 6). The change usually does not take place until after the eggs have been for some time in the uterus. As shown by the study of sections, the appearance of this pit usually coincides with the time of formation of the second polar spindle.

B. CAPACITY OF UTERINE EGGS FOR FERTILIZATION

To test whether eggs newly arrived in the uterus are capable of fertilization, a female was taken in which only a small portion of the eggs had reached the uteri, the others being distributed all along the route from ovary to uterus. The eggs from one uterus—about 75 in number—were mixed with milt after the usual manner in artificial fertilization. Of the entire lot, not a single egg developed.

In another female nearly all the eggs had arrived in the uteri, a few remaining in the oviducts and body cavity, and none in the ovaries. All the eggs from the uteri were mixed with milt; about 5 per cent of them developed.

In a third female all the eggs were in the uteri, but none of them showed a distinct pit at the animal pole—evidence that they had only recently entered the uterus. All the eggs were mixed with milt; none of them developed. It should be noted that this female was evidently in the first year of sexual maturity and the eggs may have been slow in undergoing maturation changes, or defective in some way.

In the great majority of cases of females taken with all the eggs in the uteri, artificial fertilization has been successfully performed; a high percentage of fertilized eggs is reached when all the eggs show a distinct pit at the animal pole. In every case in which seminal fluid was examined under the microscope during the breeding season, the spermatozoa were motile; so it is not likely that any cases of failure in artificial fertilization were due to defective spermatozoa.

The evidence indicates that the eggs are incapable of fertilization at the time when the first eggs reach the uterus, but that about the time all the eggs reach the uterus the majority of them become capable of fertilization. This change in their potentiality coincides in time with, or slightly precedes, the formation of a distinct pit at the animal pole; it is probably correlated with the formation of the second polar spindle (see section V).

C. CHANGES VISIBLE FROM THE SURFACE DURING FERTILIZATION

The appearance of the blastodisc shortly after fertilization is shown in figs. 7 and 8. During the first eight hours after fertilization there is an increase in the extent of the blastodisc from a diameter of 90° to 130°–160°, with a corresponding increase in the intensity of its differentiation. From this time up to first cleavage there is no constant increase in the extent of the blastodisc, though the transition from the blastodisc to the darker region surrounding the vegetal pole becomes more gradual. The pit at the animal pole persists unchanged almost up to the time of first cleavage; it is sometimes double (see fig. 10). Shortly before first cleavage it becomes broader and shallower, and usually disappears before the beginning of the first cleavage furrow.

As early as fifteen minutes after artificial fertilization, pits or scars made by the actual or attempted entrance of a spermatozoon have been found on the surface of the egg. It seems remarkable that the spermatozoon can pierce through the thick and tough gelatinous capsule in so short a time. In living material, the point of actual or attempted entrance of a spermatozoon is often visible as a minute but sharply-defined pit, barely visible to the naked eye; hence the name 'sperm pit' will be used to designate the precise locality where the spermatozoon enters, though the word 'pit' does not always accurately describe the appearance in preserved material.

The sperm pits are best studied in material killed in the bichromate-acetic-formalin mixture and preserved in formalin. The 'pits' are not all alike, but readily fall into the following classes, which probably represent consecutive stages in the penetration of the egg by the spermatozoon (see fig. 8):

(a). A simple pit, deep and sharply defined, as observed in living material.

(b). The pit is surrounded by a very small circular opaque white spot.

(c). The pit has disappeared, and the white spot remains. This type is most numerous. (Rarely, the pit persists until much later—see fig. 10.)

(d). The white spot is surrounded and sharply limited by a dark circular line.

(e). The white spot is surrounded by two concentric circular lines separated by a narrow space which is darker than the general surface of the egg (best shown in fig. 7).

It is not always possible to tell from surface views whether the spermatozoon has actually entered the egg, but from the study of

Fig. 7 Equatorial view of an egg of Cryptobranchus allegheniensis, 15 minutes after fertilization, showing a single sperm pit. The lightly stippled area in the upper part of the figure indicates the extent of the blastodisc.

Fig. 8 Equatorial view of an egg 45 minutes after fertilization, showing numerous sperm pits.

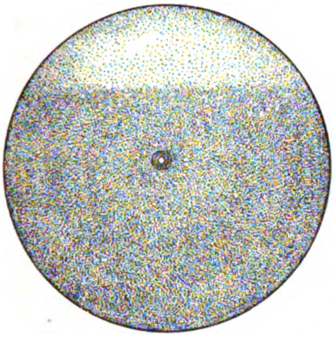
Fig. 9 View of the animal hemisphere of an egg 3¾ hours after fertilization, showing a sperm area near the edge of the blastodisc.

Fig. 10 View of the animal hemisphere of an egg 3¾ hours after fertilization, showing a later stage in the history of the sperm area. The boundary of the sperm area is a trifle too conspicuous in the figure.

Fig. 11 Equatorial view of an egg 6½ hours after fertilization, showing further extension of the sperm area.

Fig. 12 View of the animal hemisphere of an egg 7½ hours after fertilization, showing two sperm areas, on opposite sides of the blastodisc.

All the figures are drawn from preserved material. × 7.



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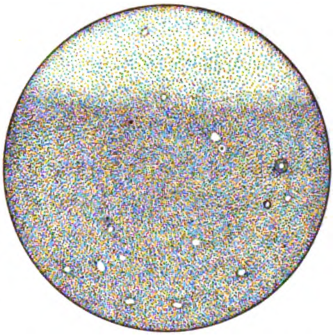

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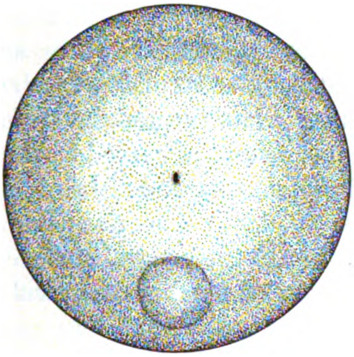

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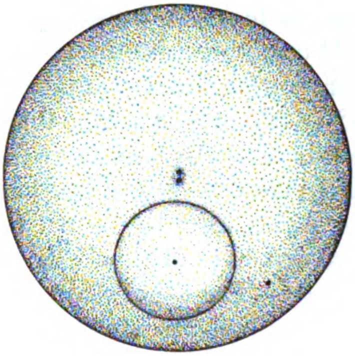

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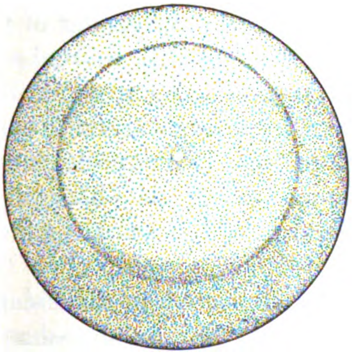

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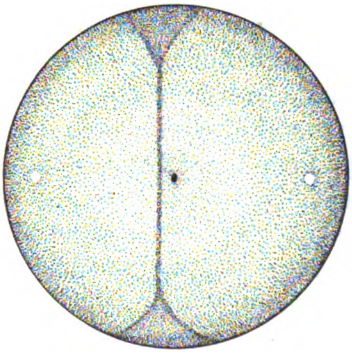

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sections it appears that the first three types of sperm pits indicate that the spermatozoon has barely penetrated through the cell wall, or that the attempt is an abortive one; the last two types indicate with considerable certainty that the spermatozoon has penetrated well into the egg.

Polyspermy is the rule. Cases of penetration by more than one spermatozoon have been found fifteen minutes after fertilization, while the surface of the egg may be scarred by a dozen or more wounds presumably made by other spermatozoa. An hour later, the majority of the eggs have been penetrated each by from one to ten spermatozoa, and sometimes scarred by as many as fifty more. In one case observed the entire number of sperm pits reached nearly a hundred.

About three hours after fertilization the small white spot representing the sperm pit is surrounded by a circular area about 10° to 15° in diameter, slightly darker than the general surface (see fig. 9). An hour later this area has increased in size, is whiter throughout its central portion, and is sharply bounded by a dark line which forms a perfect circle (see fig. 10). This dark line is, partly at least, due to a slight depression in the general surface of the egg. For convenience the area enclosed by this circle will be called the 'sperm area.'

During the next few hours the sperm area increases in size until it covers almost an entire hemisphere (figs. 11 and 12). Its surface is now in general a trifle paler than the remainder of the egg outside the blastodisc; its boundary may pass the animal pole without interruption. Two or even three sperm areas in this advanced stage may be present, their boundaries usually overlapping. Fig. 12 shows an egg fertilized from two opposite sides, the spermatozoa entering near the margin of the blastodisc; the two sperm areas meet at the animal pole, but remain widely separated in the lower hemisphere.

In monospermic eggs preserved and dissected in this stage, the sperm area is found to overlie a lenticular or disc-shaped mass, of firmer consistency than the remainder of the egg which may sometimes be shelled off in a few concentric layers like the fleshy part of an onion. Outside the boundaries of both sperm area and blastodisc there is left a crescentic area which retains the usual color of the heavily yolk-laden portions of the egg; this region often shows numerous fissures in the yolk, running parallel to the margin of the sperm area. These fissures separate the layers previously mentioned. In position and outline this area corresponds very nearly to the 'gray crescent' of the frog's egg (Roux, '83, '85, '87 and '03; Schultze, '00; see also Jenkinson, '09, p. 80 and fig. 43).

Within eight to twelve hours after fertilization the sperm pits have become indistinct and, as a rule, they all disappear before the first cleavage, though cases have been found as late as the fifth cleavage stage. Meanwhile the sperm areas also become indistinct, losing the dark line which serves as a boundary and gradually blending with the surrounding surface of the egg. Fifteen or twenty hours after fertilization, it is usually impossible to orient the egg with respect to the point of entrance of a spermatozoon; before the egg is ready for first cleavage it has resumed the general appearance of radial symmetry which it had before fertilization.

The sperm areas have not been observed in living material, but the examination was made without the aid of a binocular microscope, an instrument which has proved of great value in the surface study of the fertilization stage with preserved material.

In preserved material a space sometimes appears between the blastodisc and the vitelline membrane which elsewhere closely invests the egg. An examination of living eggs at intervals from fertilization to first cleavage shows that normally the vitelline membrane fits closely about the entire egg. The condition noted in preserved material is due to the subsidence of the blastodisc; the vitelline membrane does not spring away from the egg after fertilization, as occurs in some lower forms.

If one remove an unfertilized egg from its gelatinous envelope and immerse it in water, and place almost in contact with it a drop of seminal fluid, one observes that the spermatozoa by means of slow writhing movements disperse gradually in all directions. There is no evidence of attraction by the egg, but spermatozoa coming in chance contact with it adhere to its surface, so that in time there are more spermatozoa at the surface of the egg than at a little distance from it. As previously noted, spermatozoa are found in capsules that do not contain eggs; in this case there is no possibility of attraction by the egg.

In Cryptobranchus, as in other amphibian eggs, there is no preformed micropyle. In eggs fertilized in a natural manner, the spermatozoon may enter the egg at any point. More sperm pits have been found in the marginal region of the blastodisc, about midway between the equator and the animal pole, than elsewhere, indicating that this zone may be especially favorable to the entrance of the spermatozoon; but if any selective influence is at work, it cannot be a strong one, for spermatozoa have been found penetrating the egg close to the second polar spindle, and at various points in the lower hemisphere, even at the vegetal pole. Sperm areas are best developed about those sperm pits that occur near the margin of the blastodisc. In only one case has a sperm pit at the vegetal pole been found surrounded by a sperm area. Sperm pits are often more numerous on one side of the egg than on the opposite side, indicating a chance inequality in the exposure of the egg to the seminal fluid.

All the statements in this section regarding penetration of the egg by the spermatozoa have been confirmed by sectioning eggs which have first been carefully described externally.

D. SUMMARY

A germinal area is first visible in the ovarian egg taken about the middle of August. The germinal area is usually situated on the more exposed side of the egg, toward the periphery of the ovary; it has at first a diameter of about 60°, and increases gradually in size until about the time of first cleavage; it has then a diameter of about 145°.

In ovarian eggs examined about the first of September, the germinal vesicle is usually visible at the surface, in the center of the blastodisc; it disappears shortly before the egg leaves the ovary.

Soon after the eggs have reached the uterus, a sharply-defined pit appears at the animal pole; this pit persists up to the time of first cleavage. About the time of the appearance of the pit at the animal pole, the egg becomes capable of fertilization.

The point of entrance of a spermatozoon (the 'sperm pit') is easily recognizable in both living and preserved eggs. In preserved material, the influence of the spermatozoon on the egg substance is indicated in surface views by the differentiation of a large circular area (the 'sperm area') surrounding the sperm pit. This area is recognizable by a slight difference in color and by the presence of a bounding dark line; it increases in size until it covers nearly a hemisphere of the egg, then disappears.

In artificially fertilized eggs, and presumably in eggs fertilized in nature, polyspermy usually occurs.

There is no evidence of attraction of the spermatozoon by the egg.

The spermatozoon may enter the egg at any point, but sperm areas are best developed about those sperm pits that occur near the margin of the blastodisc.

V. THE INTERNAL HISTORY OF THE EGG BEFORE CLEAVAGE

The present section deals with a few features concerned in ovogenesis and maturation, and gives a more detailed account of the fertilization phenomena.

A. OVOGENESIS

The material for this study consists as follows:

(a). For the early stages it was found best to use larval and immature post-larval females, with a body-length ranging from 9 to 38 cm. (two years old and upward). Females with a body length of more than 38 cm. are almost always sexually mature.

(b). The residual eggs of spent females taken in September furnished ovocytes slightly older than those of the largest immature females taken in August and September.

(c). Mature females taken during July and August furnished material for the late stages of ovogenesis.

There remains a period during the last year of development, extending from October to June inclusive, which is not represented in the material. Since during this time, which includes the winter months, development is least active, the lack of these stages is of minor importance for the purposes of the present paper.

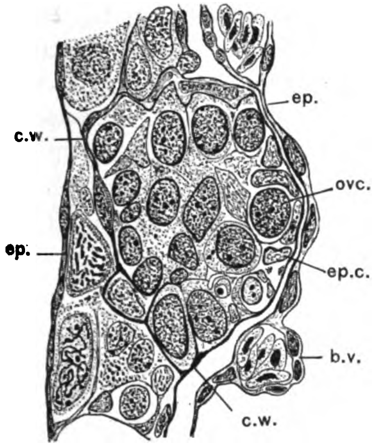
1. The formation of the follicle and the egg membranes

The young ovary of Cryptobranchus is essentially a sac with thick cellular walls. In a 9 cm. larva the ovarian wall (see figs. 13 to 17) shows structural differentiation as follows: (*a*) an inner and an outer limiting membrane of flattened epithelium; these membranes are connected by (*b*) a network of cells of a character similar to those comprising the limiting membranes, though usually not so greatly flattened; within the meshes of this network are found (*c*) young ovocytes in various stages of development.

In the ovary of a 9 cm. larva, more or less clearly defined groups or cysts of very young ovocytes (see fig. 13) may be found, each group surrounded by a thin epithelial membrane, the cyst membrane. All the ovocytes of each group or cyst are presumably the product of a single primary ovogonium. Epithelial cells also occur within the cyst. Within many of these cysts, development has gone further, and some or perhaps all the ovocytes have undergone an increase in size which involves both nucleus and cytoplasm (see fig. 14). Within each cyst, one ovocyte usually outstrips its fellows, and becomes surrounded by a layer of epithelial cells which form the follicle (fig. 15).

With a further increase in size of the ovocyte, the follicular layer assumes the character of a definite membrane with somewhat flattened cells, and that portion of the cyst membrane in contact with the ovarian membrane shows an increase in the number of its nuclei and is more clearly differentiated as a separate layer (see fig. 16).

With a still greater increase in size, as shown by the most advanced ovocytes of a 9 cm. larva and in later stages, the egg presses the overlying membranes into the central cavity of the ovary, so that the ovocyte comes to be suspended as in a sac, and is more nearly surrounded by the cyst and ovarian membranes (see figs. 17 to 21). In all three membranes, an increase in the



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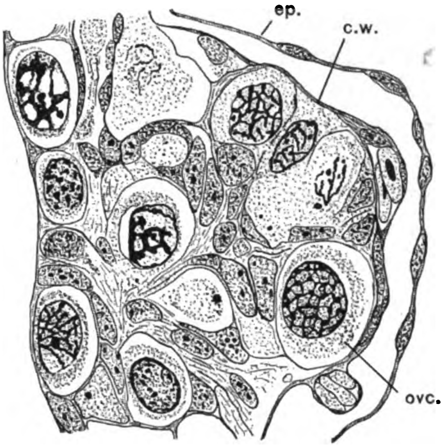

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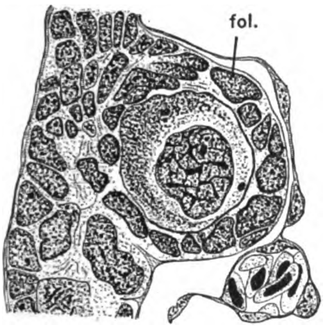

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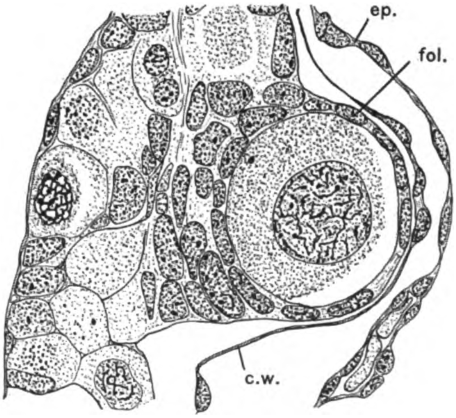

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Figs. 13 to 16 Cross-sections through the wall of the ovary of a 9 cm. larva of Cryptobranchus alleghenieneis. × 300. *b. v.*, blood vessel; *c. w.*, cyst wall; *ep.* (right), inner epithelial membrane of the ovarian wall; *ep.* (left), outer epithelial membrane of the ovarian wall; *ep. c.*, epithelial cell of the cyst; *fol.*, follicle cell; *ovc.*, ovocyte.

Fig. 13 A cyst containing young ovocytes and epithelial cells occupies the central part of the figure.

Fig. 14 A cyst containing slightly older ovocytes.

Fig. 15 An ovocyte surrounded by the newly-formed follicle.

Fig. 16 An ovocyte and follicle slightly more advanced than the one shown in preceding figure.

number of nuclei keeps pace with the increase in extent. In a 35 cm. female (see figs. 21 and 22), the nuclei of the follicular membrane are the most numerous and least flattened; those of the cyst membrane and inner ovarian membrane are both decidedly flattened. Somewhat rarely, the cyst membrane is ruptured by the expansion of the ovocyte. According to King ('08) in Bufo the rupture of the cyst membrane takes place regularly at an early stage.

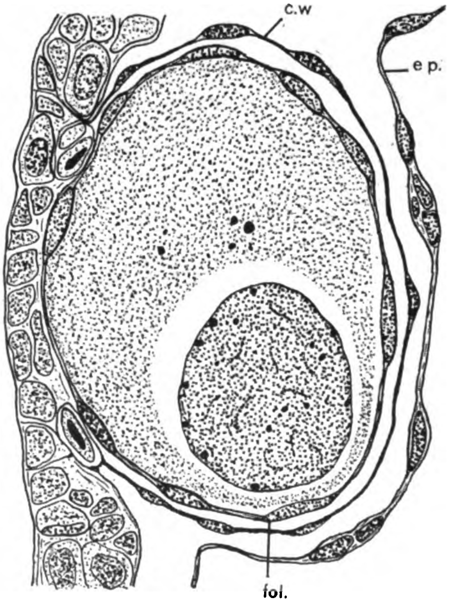


Fig. 17 Cross-section through the ovarian wall of a 9 cm. larva of Cryptobranchus allegheniensis, showing one of the most advanced ovocytes. × 300. Lettering as in the preceding figures.

The ovocyte in the advanced growth stage is thus surrounded by a single-layered follicle, suspended in a flask-shaped two-layered sac of which the inner layer is the cyst membrane, the outer layer is the inner epithelial membrane of the ovarian wall. In a broader sense, the entire three-layered structure may be called a follicle, and the neck of the flask-shaped sac may be called the stalk of the follicle. This triple-layered wall persists without any radical change in structure up to the time of maturation.

In the later stages of the development of the ovary, its walls anastomose by the formation of cross-walls or partitions, dividing the ovary into compartments or perhaps pockets; by these cross-walls the course of the inner ovarian membrane is greatly complicated.

The ovocyte of a female of 26 cm. and younger is apparently a naked cell, possessing no proper membrane. In females with a body length of from 30 to 35 cm. there occurs a rapid development of two non-cellular membranes closely investing the egg within the follicle. The inner of these two membranes exhibits a radial striation and is the zona radiata; at the time of maturation it becomes a simple cell wall to the egg. The outer membrane, clear and homogeneous, is the zona pellucida; it persists as the 'vitelline membrane' of the embryo.

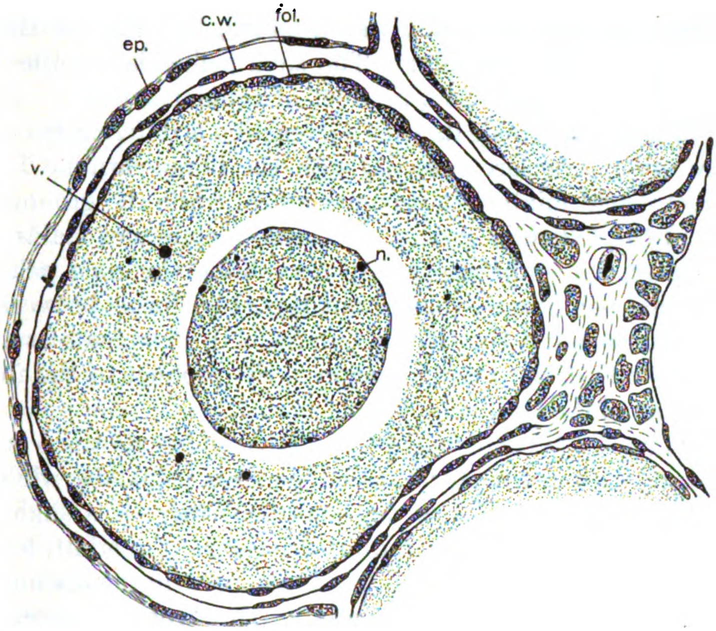


Fig. 18 Cross-section through the ovarian wall of a 26 cm. Cryptobranchus alleghenienis, showing one of the most advanced ovocytes. × 180. *n.*, nucleolus; *v.*, vitelline body; *ep.*, inner epithelial membrane of the ovarian wall. Other lettering as in figs. 13 to 16.

The zona radiata and the zona pellucida begin to form simultaneously, shortly before the appearance of yolk granules. In the most advanced ovocytes of a 35 cm. female, these membranes are well established and a narrow zone of yolk has appeared near the periphery of the ovocyte (see fig. 22).

The zona radiata arises from the peripheral cytoplasm of the ovocyte. In its early stages its inner boundary is not sharply defined; its staining reaction is like that of the egg cytoplasm; aside from its cross-striation its structure, like that of the egg cytoplasm, is finely granular. The zona pellucida, on the other hand, is formed *de novo* as a product of cellular activity. In the ovary of a 35 cm. female its staining reaction is different from that of any other structure present: with the borax-carmine Lyons-blue picric-acid mixture it becomes green, while the ground-substance of the follicular, cyst and ovarian membranes stains blue. Since, later, a membrane exactly resembling the zona pellucida in character sometimes, though not typically, forms between the cyst membrane and the follicle (see fig. 32), it seems reasonable to conclude that the zona pellucida is the product of the follicle rather than of the egg.

In the most advanced ovocytes of a spent female there is usually an increase in the thickness of the zona pellucida, while the zona radiata shows signs of degeneration—there is a slight loss in the distinctness of the radial striations. In adult females taken in July and August, there is a further loss in the distinctness of the striations of the zona radiata. In ovocytes taken just before maturation, with the germinal vesicle close to the surface, the zona radiata has in some cases almost lost its radial striation, is decreased in thickness, and is becoming a simple cell wall to the egg.

The literature on the zona pellucida and zona radiata of the amphibian egg has been reviewed by Waldeyer in Hertwig's ('06) Handbuch and needs no summary here.

The ovary of a young Necturus 20 cm. long, killed August 25, gives stages corresponding to those of a 35 cm. Cryptobranchus. The follicular layers and mode of attachment of the ovocyte to the ovarian wall are practically the same as in Cryptobranchus, with the exception that there is a marked difference in the appearance of the nuclei of the follicle proper: in Necturus these nuclei are more numerous, and in form are spherical or even elongated in a radial direction, instead of being flattened in the direction of the circumference of the egg as in Cryptobranchus. The follicle of Necturus more closely resembles that of the selachian egg in an early stage (see Hertwig's Handbuch, '06, figs. 105 and 195). The zona pellucida and zona radiata are much alike in the two urodeles; the striations of the latter membrane are rather more distinct in Necturus.

2. The establishment of polarity, and the progress of axial differentiation

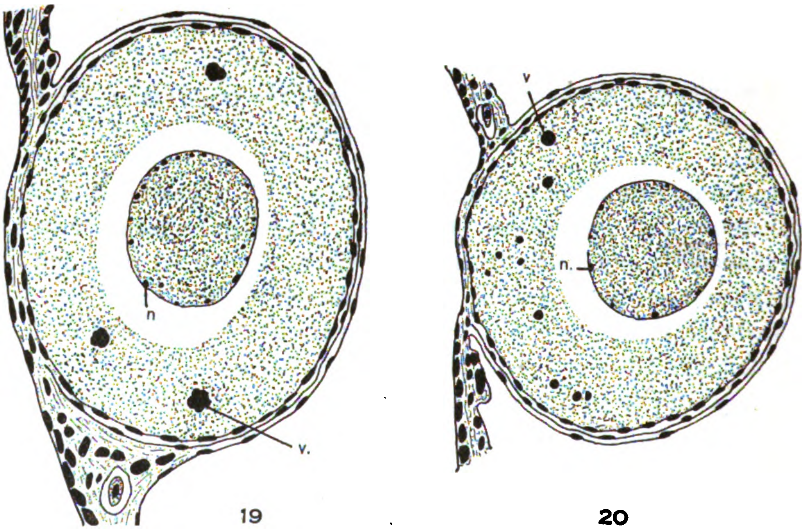
As already noted in the surface study of the ovarian egg, the ovocyte ready for maturation shows its telolecithal character in the presence of a superficial germinal area, in the center of which lies the germinal vesicle, while the remainder of the egg is heavily laden with yolk. It is the purpose of the present section to trace the changes by which this axial differentiation is brought about.

In the ovary of a 9 cm. larva, vitelline bodies (see King, '08) are recognizable in the cytoplasm of the ovocytes in all stages present, but are not very numerous nor conspicuous even in the most advanced ovocytes of such an ovary (see figs. 13 to 17). In the largest ovocytes, the germinal vesicle is usually somewhat excentrically situated, but with no constancy in the direction of excentricity. Faintly-staining nucleoli are distributed quite promiscuously thoughout the germinal vesicle, in the later stages with a slight tendency toward forming a ring at the periphery.

In the most advanced ovocytes of a 26 cm. female (see fig. 18) there is less excentricity in the position of the germinal vesicle; the nucleoli are most numerous at the periphery. There is an increase in the number and size of the vitelline bodies, which are more numerous on the side toward the central cavity of the ovary. After fixation in Zenker's fluid, both nucleoli and vitelline bodies take the nuclear stain, though faintly. In the ovary of a 27 cm.

female, fixed in Bouin's solution, the nucleoli take the nuclear stain very faintly; the vitelline bodies take the cytoplasmic stain.

In the most advanced ovocytes of a 30 cm. female (figs. 19 and 20) the germinal vesicle is quite centrally situated—a position which it retains until a very late stage of ovogenesis. The nucleoli, which still stain but faintly, are nearly all at the periphery, where they form a uniform ring. The vitelline bodies shown in the figures now stain brilliantly with borax carmine used after Zenker's fluid; in general they are much more numerous on the side toward the periphery of the ovary, in the region of the future animal pole. Some of the vitelline bodies are very large; these usually occupy an equatorial position, but are sometimes found on the inner side of the ovocyte. Comparison with the preceding stage suggests that the vitelline bodies originate on the inner side of the ovocyte and migrate to the outer side; that they reach their greatest development midway in the course of migration, and break up to form the smaller and more numerous vitelline bodies



Figs. 19 and 20 Sections through ovocytes and ovarian wall of a 30 cm. Cryptobranchus allegheniensis, showing the follicle and the distribution of vitelline bodies and nucleoli. × 90. *n.*, nucleolus; *v.*, vitelline bodies.

in the region of the future animal pole. But scattered throughout the cytoplasm are occasionally to be found other bodies, resembling the vitelline bodies but more irregular in form and staining very faintly. While it is possible that these bodies are different in kind from the brilliantly-staining vitelline bodies, their appearance suggests that they are stages in the degeneration of the latter. The faintly-staining bodies, though seldom numerous, are more frequently found in regions poor in deeply-staining vitelline bodies. These observations enable us to offer an explanation of the distribution of vitelline bodies, alternative to the theory of migration: a wave of development of vitelline bodies, followed by a wave of degeneration, may sweep from the inner to the outer hemisphere of the ovocyte. But whether migration is real or only apparent, the fact remains that the region of most abundant deeply staining vitelline bodies has shifted from the vicinity of the future vegetal to the future animal pole of the ovocyte. This change is perhaps an expression of polarity; if so, it is the first indication of polarity that I have observed. However, it is not at all certain that polarity is not present at an earlier period; in particular the history of the chromatin has not been sufficiently studied, moreover it is of course possible that a physiological polarity of the cell may precede its manifestation in a visible form.

In the ovary of a 34 cm. female, fixed in Flemming's solution, the distribution of vitelline bodies is much the same as noted in the 30 cm. female; in form the vitelline bodies are sometimes oval or irregular, but never mulberry-shaped as is sometimes the case with Zenker's.

In the ovary of a 35 cm. female, yolk granules are beginning to form in the most advanced ovocytes; other ovocytes nearly as large contain no yolk. In neither of these two stages are vitelline bodies in the typical condition present, but they are sometimes found undergoing a process of degeneration—they lose the intensity of their staining reaction, become irregular in form, and disappear. The disappearance of the vitelline bodies at the time of the formation of yolk suggests a correlation between the two phenomena; but so far as I have been able to observe, the final stages in the disappearance of the vitelline bodies are not closely associated with the formation of yolk granules, nor have I found any undoubted 'yolk nuclei,' such as have been described by King ('08) for Bufo. In view of the diversity in the methods of yolk-formation described for different amphibians, this result is not altogether surprising.

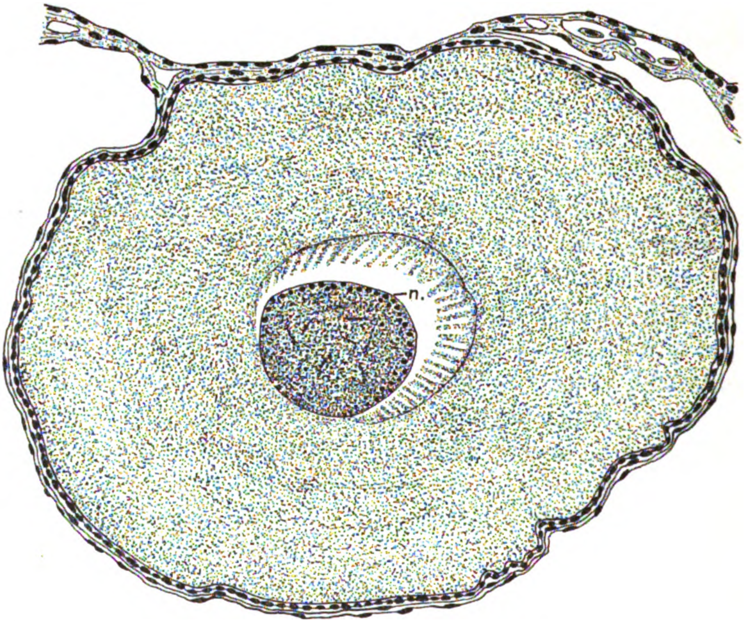


Fig. 21 Section through an ovocyte and ovarian wall of a 35 cm. Cryptobranchus allegheniensis, showing the follicle and the distribution of nucleoli. In this ovocyte the vitelline bodies have disappeared, but yolk-formation has not yet begun. × 60. *n.*, nucleolus.

In the more advanced ovocytes of a 35 cm. female (see fig. 21). the nucleoli stain deeply with borax carmine used after Zenker's fluid. There is usually a marked concentration of the nucleoli on the side of the germinal vesicle toward the periphery of the ovary. Account must be taken of the fact that shrinkage of the germinal vesicle also proceeds, as a rule, most extensively on this side, leaving a large space, while the opposite side remains in contact with the cytoplasm. This greater shrinkage on the outer side in part accounts for the greater concentration of nucleoli on this side, but it is inadequate to account for all of it; moreover the axis of excentricity in form due to shrinkage does not always correspond accurately to the axis of excentricity in the arrangement of the nucleoli.

This excentric distribution of material marks an axis which corresponds, roughly at least, to the polar axis at the time of maturation; the nucleoli accumulate on the side which is to become the animal pole, and thus perhaps afford a second indication of polarity. King ('08) found this condition in Bufo at the time when the nucleus was moving from the center of the egg to the animal pole, and suggested the possibility that the accumulation of most of the nucleoli in one part of the nucleus might have something to do with this movement. In Cryptobranchus this concentration of the nucleoli begins long before the migration of the germinal vesicle to the surface, and indeed before the formation of any yolk; it is most marked in the advanced ovocytes of a 35 cm. female, when the yolk is just beginning to form. As will appear from the study of later stages, this arrangement of the nucleoli does not persist during the actual migration of the germinal vesicle; nevertheless the early occurrence of axial concentration of nucleoli is significant.

In the ovary of a 35 cm. female, we find that occasionally, through the folding of the ovarian wall, an ovocyte has been thrust deep into the central cavity and has come in contact with the nutrient ovarian wall of the opposite side. The side opposite the stalk of the follicle now becomes the side best nourished, and here the nucleoli accumulate. Thus nature's experiment shows that the accumulation of nucleoli, and perhaps polarity, is not something predetermined in the egg, or even fixed by the relation of the egg to the ovarian wall within which it develops, but is a phenomenon depending upon larger environmental relations which probably have to do with nutrition; for as a consequence of the changed position of the egg the nucleoli accumulate on the opposite side from that favored by the original environment.

In the ovocytes of immature females with body lengths of from 35 to 38 cm., the yolk first appears in a narrow zone near the periphery and parallel to the newly-formed zona radiata, but separated from the latter by a narrow layer of clear cytoplasm (see fig. 22). At this time the ovocyte has a diameter of from 1.5 mm. to 2 mm. The yolk zone is divisible into two layers, an outer layer of fine yolk particles and an inner layer of coarse yolk particles, separated by a narrow region poor in yolk.

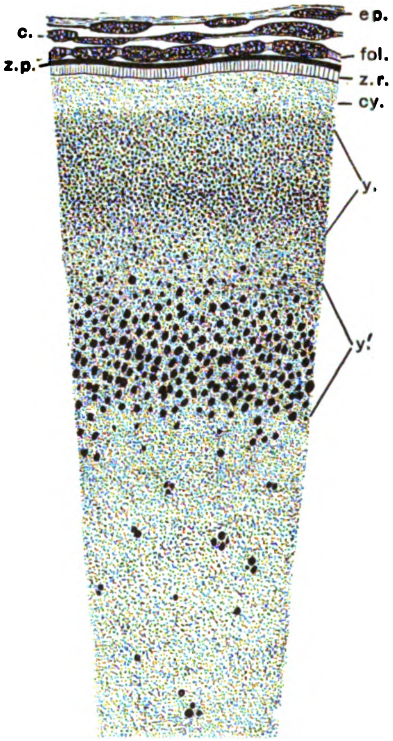


Fig. 22 Portion of a section through one of the most advanced ovocytes of a 35 cm. Cryptobranchus allegheniensis, showing structure of the membranes surrounding the egg and the distribution of yolk granules. × 340. The strip shown extends about half-way to the germinal vesicle. *c.*, cyst membrane; *cy.*, yolk-free peripheral zone of cytoplasm; *ep.*, inner epithelial membrane of the ovarian wall; *fol.*, follicular membrane proper; *z. p.*, zona pellucida; *z. r.*, zona radiata; *y.* and *y'.*, layers of fine and coarse yolk granules respectively.

In the largest residual eggs (2 to 3 mm. in diameter) of spent females, the yolk-laden zone has extended inward further than outward; a very narrow zone of clear cytoplasm persists at the periphery, and a much broader zone containing only a few scattering yolk granules surrounds the germinal vesicle. The middle portion of the yolk zone is now filled with coarse yolk granules; its margins consist of fine yolk particles. The germinal vesicle is still centrally situated, and the arrangement of yolk zones and cytoplasm is concentric. The nucleoli are still distributed at the periphery of the germinal vesicle, with only a slight tendency toward concentration at the outer side.

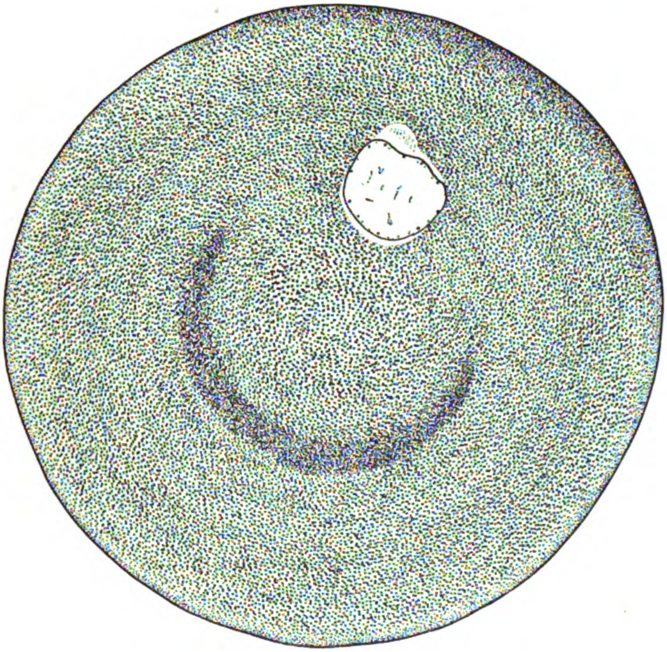


Fig. 23 Meridional section through one of the most advanced ovocytes of an adult Cryptobranchus allegheniensis killed July 6. The bounding line represents the zona radiata. × 20.

In the largest ovocytes of adults killed July 6 (see fig. 23), the germinal vesicle occupies a position midway between the center of the egg and the periphery, on the more exposed side of the egg, toward the stalk of the follicle. The animal pole is thus defined by a point on the surface, toward which the germinal vesicle is moving. The cytoplasm is now everywhere thickly interspersed with yolk granules; these granules are in general coarse throughout the central portion of the egg, finer and more densely packed at the periphery. Axial differentiation in the arrangement of yolk particles is now for the first time evident in a slight thickening of the peripheral layer of fine yolk particles in the region of the animal pole. This region is also somewhat richer in cytoplasm than the remainder of the egg. There is thus present the beginning of a germinal disc or blastodisc, which in later stages becomes visible in surface views as the germinal area.

In the vegetal hemisphere a region of particularly fine and dense yolk, crescent-shaped in meridional section, lies mid-way between the center of the egg and the periphery. This region I shall call the 'yolk cup.' Its appearance suggests that it may be a part of a once continuous zone completely enclosing the germinal vesicle, and that, in the animal hemisphere, this zone has been interrupted in consequence of the migration of the germinal vesicle toward the surface. Probably the yolk cup is the physiological equivalent of the concentric layers of dense fine yolk found in the egg of the hen and various other vertebrates. Riddle ('11) has shown that the alternate layers of yellow and white yolk in the hen's egg are due to a daily rhythm in nutrition; he has advanced the same principle in explanation of the concentric layers of yolk in the eggs of certain cyclostomes, selachians and reptiles. In Cryptobranchus, from comparison with ovarian eggs taken in the autumn after the close of the spawning season, it is evident that in the stage under consideration the yolk cup marks the limits of growth during the preceding winter; hence it seems very probable that the yolk cup is the result of a seasonal variation in nutrition, and represents a layer added during the winter months.

The nucleoli are still found mainly at the periphery of the germinal vesicle, but with no constant tendency toward concentration in an axial position.

It has been noted that the animal pole, as defined both by the center of the germinal disc and the point on the surface toward which the germinal vesicle is moving, lies in general on the more exposed side of the egg, within the stalk of the follicle. The animal pole thus lies in the opposite direction from that assumed in the ovarian egg of the hen (Lillie, '08, p. 29). According to King ('02) in the great majority of cases the egg of Bufo is attached in the equatorial region by the stalk of the follicle.

From a comparison of this stage with the preceding one (the ovocytes of a spent female), it is evident that yolk-formation proceeds concentrically about a centrally situated germinal vesicle until the egg is nearly or quite filled with yolk, and that axial differentiation in the arrangement of yolk particles does not appear until a very late stage of ovogenesis, two or three months before maturation. It is further apparent that the germinal vesicle attains its final position, not through unequal growth of the cytoplasm or excessive accumulation of yolk on the other side of the egg, but by a process of migration.

In the ovocytes of adults taken July 20, the germinal vesicle has migrated further toward the animal pole; it lies about one-third of the distance from the surface to the center of the egg. Both nucleoli and chromosomes are now aggregated at the center of the germinal vesicle. The yolk-cup persists, and there is an increase in the extent of the germinal disc. In some eggs a small cone-shaped mass of dense cytoplasm, with the apex of the cone pointing inward, lies immediately beneath the germinal vesicle.

In the ovary of an adult female killed August 17, the egg (fig. 24) has nearly reached its maximum size before fertilization; a meridional section cut in paraffin has a diameter of about 6 mm. (It should be noted that a yolk-laden egg does not shrink in paraffin to the same extent as ordinary tissues). The germinal vesicle lies only a short distance from the surface, and is bounded on the side toward the center of the egg by a large cone-shaped mass of cytoplasm. The apex of this cone is continuous with a slender meshwork of less dense but yolk-free cytoplasm extending half-way to the center of the egg. Owing to a slight obliquity of the slender cytoplasmic mass, it has not been found complete in any one section; in fig. 24 it has been added, from adjacent sections, to the one chosen for the remainder of the drawing.

Immediately beneath the zona radiata lies a peripheral layer of yolk-free cytoplasm, which from analogy with the teleost egg I shall call the 'protoplasmic mantle.' In the region of the vegetal pole this is so thin as to be barely recognizable with a magnification of 500 diameters; in the region of the animal pole it is thickened to form a disc which I shall call the 'cytodisc.' At the animal pole the cytodisc reaches its maximum thickness of about 15μ—a little thicker than the layer of follicle cells proper.

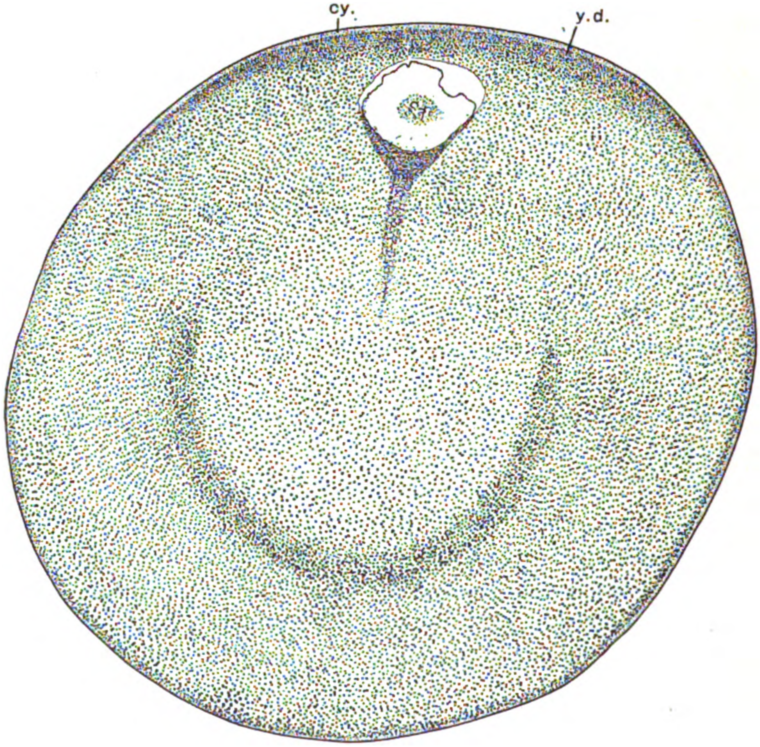


Fig. 24 Meridional section through an ovarian egg of an adult Cryptobranchus allegheniensis killed Aug. 17. × 20. *cy.*, cytodisc; *y. d.*, yolk disc.

The remainder of the egg is filled with yolk. Underlying the cytodisc and occupying an area about 100° in diameter surrounding the animal pole, is a thick layer of fine but dense yolk which I shall call the 'yolk disc.' The cytodisc and yolk disc combined represent the anlage of the germinal disc or blastodisc, which later comes to enclose the germinal vesicle and the cytoplasm accumulated beneath it. Elsewhere a very thin peripheral layer of fine yolk particles, continuous with the yolk disc, lies immediately beneath the protoplasmic mantle. The interior of the egg shows no particular change in the yolk.

The germinal vesicle is spherical when perfectly preserved; when flattened this is due to shrinkage. The ground-substance or nuclear sap appears homogeneous under a low power, but with a magnification of 500 diameters it exhibits an extremely fine but dense granular structure. The nucleoli are now nearly all aggregated at the center; some few persist at the periphery, particularly on the side toward the center of the egg. The chromosomes are for the most part confined to the central part of the area occupied by the nucleoli.

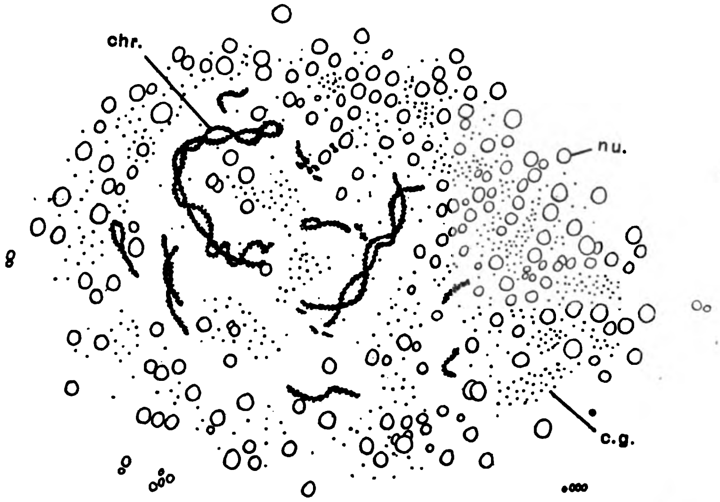


Fig. 25 Central portion of the germinal vesicle represented in the preceding figure, enlarged to show details. The finely granular ground-substance of the germinal vesicle is not shown. × 340. *c. g.*, chromatin granules; *chr.*, chromosomes; *nu.*, nucleolus.

Among the nucleoli, though not closely associated with them, there are now found very numerous and minute granules which stain like chromatin (see fig. 25). These are evidently formed in close association with the chromosomes; in earlier stages chromosomes have been found covered with these granules before the latter have appeared elsewhere.

In the ovarian eggs of an adult killed August 22, the most marked changes in the general topography as viewed in meridional sections are a slight advance in the migration of the germinal vesiicle toward the surface, and an increased thickness of the peripheral zone of fine yolk particles, particularly in the yolk disc. In the vegetal hemisphere the protoplasmic mantle is no longer recognizable as a separate layer; its constituents have mingled with the peripheral layer of fine yolk particles. The cytodisc is reduced in thickness by the blending of its inner surface with the yolk disc.

The narrow path of cytoplasm leading toward the center of the egg from the apex of the cone of cytoplasm underlying the germinal vesicle has disappeared; likewise the yolk cup is, as a rule, no longer present. In this stage there is a slight increase in the number of chromatin granules dispersed amongst the nucleoli; otherwise the nuclear contents seem unchanged.

Ovaries taken during the last week in August and the first week in September usually contain some eggs with the germinal vesicle appearing at the surface. In the general organization of the egg before the germinal vesicle actually reaches the surface, there are few changes from the condition described for August 22. Fig. 26 shows the general topography of an egg with the germinal vesicle very close to the surface. The cone of cytoplasm underlying the germinal vesicle is beginning to mingle with the yolk; it is not present in the section figured. Within the germinal vesicle the nucleoli are massed more closely together at the center; there is an increase in the number of chromatin granules, and apparently a gradual disappearance of the chromosomes—in some eggs they could not be found.

At the close of the period considered, axial differentiation is evident in the following arrangement of material: (*a*) the excentric position of the germinal vesicle and the cone-shaped mass of cytoplasm underlying it; and (*b*) the formation about the animal pole of a germinal disc or blastodisc consisting of two layers, a very thin peripheral layer of yolk-free cytoplasm which has been called the cytodisc, and underlying this a thick lenticular layer of mingled cytoplasm and dense fine yolk which has been called the yolk disc. At any given level the egg is radially symmetrical about the axis of polarity. In general the egg has progressed from an alecithal through an isolecithal to a telolecithal stage.

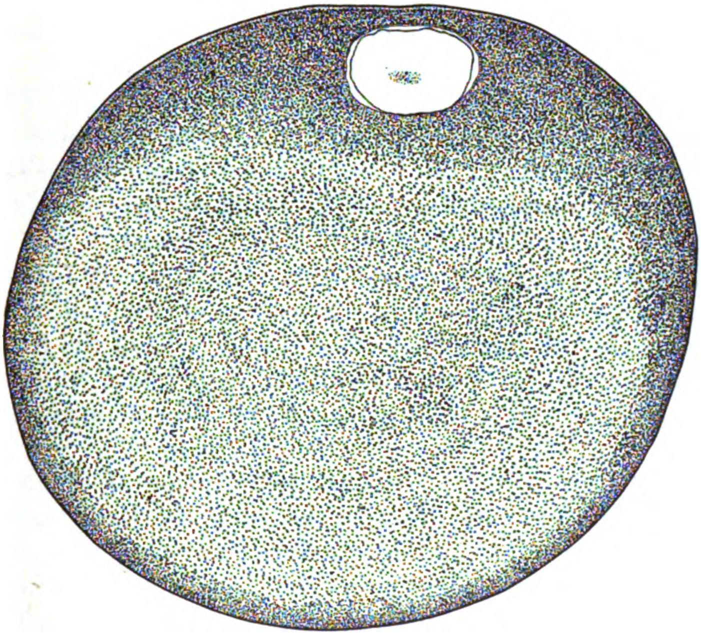


Fig. 26 Meridional section through an ovarian egg of an adult Cryptobranchus allegheniensis killed Sept. 6, 1910, showing organization just before the germinal vesicle reaches the surface. × 20.

The establishment of polarity, with axial differentiation, is an event of great morphogenetic importance, since the formative materials for the embryo are being segregated in the vicinity of the animal pole. Through later changes in the distribution of this material the animal pole comes to mark the anterior, the vegetal pole the posterior end of the future animal; hence the establishment of polarity defines the principal axis of the embryo.

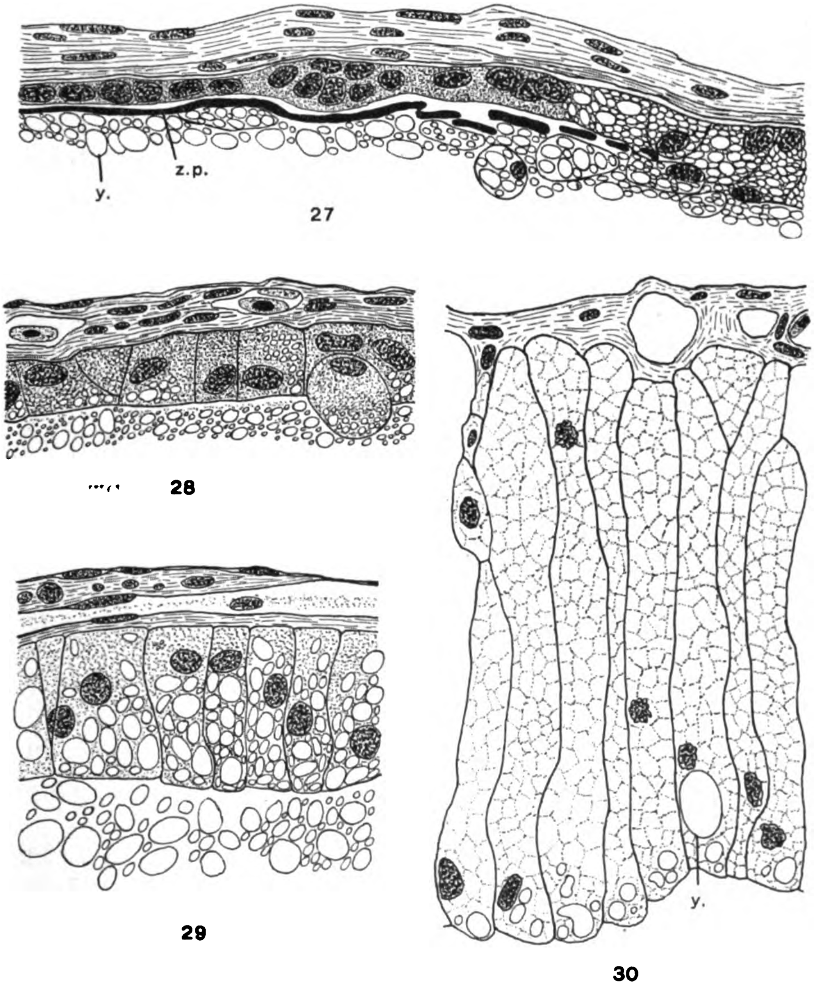
The changes that immediately follow—the appearance of the germinal vesicle at the surface, the rupture of its membrane, and the reorganization of the germinal disc with the incorporation of materials brought from the interior of the egg by the nucleus—lead up to maturation and will be considered in the account of that process.

3. Resorption of ovocytes; the follicle cells in a phagocytic rôle.

In young females nearing maturity (about 38 cm. body length), a few ovocytes reach an advanced stage of development, becoming filled with yolk and attaining a size nearly as great as the ovocytes of an adult. These precocious ovocytes fail to undergo maturation changes, and during the breeding season begin to degenerate, or rather to be resorbed, together with some of the less advanced ovocytes only partially filled with yolk. Viewed in the living ovary, these degenerating ovocytes are colored a very bright yellow or orange. Digestion and absorption of the yolk granules is accomplished through the medium of the cells of the follicular layer proper, which become greatly enlarged and function as phagocytes, thereby reversing their usual rôle as nurse cells to the egg.

The first step in the process of degeneration of the ovocyte is the disappearance of the zona radiata; the later stages are illustrated by figs. 27 to 30. The follicle cells enlarge, by increase both in the size of the nucleus and in the amount of cytoplasm. The zona pellucida is ruptured; at the same time it becomes irregularly thickened, a circumstance which may be interpreted either as a shortening of the fragments due to the release of tension, or as a step in the process of dissolution. The rupture of the zona pellucida allows the yolk to come in contact with the follicle cells; the latter engulf the yolk particles, and become surrounded by thin walls. About this time the zona pellucida disappears, and the follicle cells are left as large yolk-filled cells resembling columnar epithelium, forming a continuous layer around the egg.

Digestion of the yolk particles is completed first at the outer margin of the follicle cells, while the inner margin continues to engulf yolk. The included yolk granules stain less deeply with



Figs. 27–30 Changes at the periphery of an ovocyte in the process of resorption; the follicle cells are shown in a phagocytic rôle. Fig. 27, read from left to right, shows the beginning of the process (compare with fig. 22 showing the normal condition of the follicle). The remaining figures, taken from different ovocytes, show successively later stages. × 244. *y.*, yolk; *z. p.*, zona pellucida.

haemotoxylin than the others, and in sections stained with the borax-carmine Lyons-blue picric-acid mixture the included granules take the cytoplasmic stain more deeply.

The follicle cells later become greatly elongated, and the cytoplasm takes the form of a faint meshwork with large spaces. Ingestion of yolk continues at the inner ends of the cells, while the remainder of the cell functions as a long tube to convey the products of digestion to the periphery. The follicular layer remains one cell in thickness until the cells have reached a length of about 250 μ; with a further increase in thickness it becomes broken up into a meshwork of cells, amongst which are numerous capillaries. Ovocytes have been found in which this meshwork of cells reaches nearly to the center, and the remaining yolk is very small in amount.

In the adult female occasional eggs, though of full size, fail to escape from the ovary. Judging from their external appearance these ovocytes undergo resorption in the manner just described. A somewhat similar process of resorption has been described in the eggs of cyclostomes and fishes (Bühler, '02).

4. The organization of the egg shortly before the appearance of the germinal vesicle at the surface

At this point it may be well to summarize briefly the condition of the ovocyte in the stage immediately preceding the appearance of the germinal vesical at the surface (see fig. 26).

The egg lies within a triple-walled follicular sac whose cellular membranes have undergone little change since they first became well established. The stalk of the follicle, and in general the animal pole of the egg, lie toward the periphery of the ovary.

The zona pellucida persists unchanged, except for a slight increase in thickness; the zona radiata shows signs of atrophy, and in some cases is assuming the character of a simple cell wall.

The nucleus or germinal vesicle has migrated from the center of the egg to a position near the periphery, ordinarily on the side toward the stalk of the follicle. During the migration of the germinal vesicle a cone-shaped mass of dense cytoplasm has collected beneath it, and is now beginning to mingle with the surrounding yolk.

A germinal disc or blastodisc is evident in surface views of living material as a circular area, lighter in color than the rest of the egg, about 60° in diameter and situated on the more exposed side of the egg. In meridional sections it is shown to consist of two layers: a thin peripheral layer of cytoplasm, the cytodisc; underlying this a thick lenticular mass of mingled fine yolk particles and cytoplasm, the yolk disc. The germinal vesicle lies at the center of the yolk disc.

The yolk disc is continuous with a thin peripheral layer of fine yolk granules, mixed with cytoplasm, which lies in contact with the zona radiata everywhere except in the region of the cytodisc.

The remainder of the egg is filled with coarse yolk granules mingled with fine yolk granules and a small amount of cytoplasm.

The nucleoli are grouped at the center of the germinal vesicle, and amongst them are numerous chromatin granules. In some eggs chromosomes are found at the center of the group of nucleoli, in others the chromosomes have disappeared.

A point on the surface overlying the center of the germinal vesicle marks the animal pole. The general arrangement of materials is radially symmetrical about the axis of polarity, with differentiation proceeding in the direction of this axis.

B. MATURATION

1. The germinal vesicle at the surface

Meridional sections through ovocytes with the germinal vesicle at the surface show little change in the details of structure from the condition previously described. The germinal vesicle is usually somewhat flattened against the periphery, and a portion of its surface is in direct contact with the zona radiata. Masses of a wavy fibrous material are occasionally found in the nuclear sap. A few fragments of chromosomes are present in some eggs; in others no chromosomes have been found. The nucleoli and chromatin granules persist at the center of the germinal vesicle.

2. *The dissolution of the germinal vesicle, and the formation of the first polar spindle*

Material for the study of this stage was obtained from two females in which the majority of the ripening eggs had left the ovary, and were found distributed in the body cavity, oviduct and uterus. The nearly mature eggs left in these ovaries were found in every case investigated (nine eggs were sectioned) to have the germinal vesicle ruptured and its constituents well mixed with those of the blastodisc; in the majority of cases the first polar spindle had already formed.

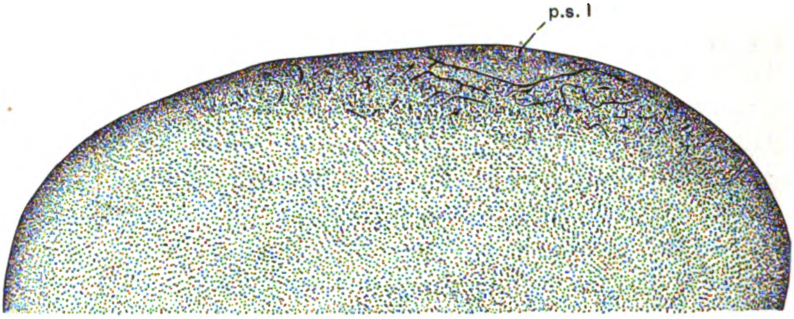


Fig. 31 Meridional section through an ovarian egg of Cryptobranchus alleheniensis, shortly after the rupture of the germinal vesicle. Fragments of the germinal vesicle are seen scattered throughout the blastodisc. × 18. *p. s. I*, first polar spindle.

The rupture of the germinal vesicle and the distribution of its materials throughout the blastodisc must take place with considerable rapidity, since in eggs sectioned only the beginning and the end of the process have been observed. Fragments of the nuclear membrane, together with the wavy fibrous material previously noted in the germinal vesicle and innumerable fine granules, probably derived from the cell sap, become widely scattered throughout the germinal disc (see fig. 31). During this process of disintegration of the germinal vesicle the nucleoli and chromatin granules are lost to view. It seems improbable that all the chromatin granules should again be segregated as nuclear material; at any rate the rupture of the germinal vesicle affords an opportunity for the contribution of important nuclear material to the cytoplasm.

The germinal disc or blastodisc no longer shows a division into two layers; the material of the cytodisc is intimately mingled with that of the yolk disc. The cone of cytoplasm following the germinal vesicle in its migration is likewise more or less thoroughly incorporated into the blastodisc.

The end result of the migration of the germinal vesicle to the surface and its disintegration in that situation is now apparent. All the material of the nucleus and a considerable amount of cytoplasm have been brought from the interior of the egg to the vicinity of the animal pole, fragmented, and the débris more or less scattered throughout the blastodisc. Out of this complex there soon emerges close to the surface at the animal pole the reconstructed nucleus in the form of the first polar spindle. One function of migration is doubtless to get this nuclear material to the periphery where a part of it may be disposed of in the maturation divisions. A further adaptation is found in the fact that, later, the egg-nucleus or female pronucleus is left in the center of the formative material of the blastodisc. A third end attained by migration is that the formative material of the blastodisc is added to by cytoplasm following the germinal vesicle, and also by substances derived from the germinal vesicle itself.

The zona radiata has become reduced in thickness, has lost its striation and no longer shows a distinct limiting inner surface—its inner margin is irregular or blends with the peripheral cytoplasm of the egg. When the egg is shrunken away from the zona pellucida the zona radiata usually remains organically connected with the egg. The character of the zona radiata has changed so radically that it will no longer be referred to by this name; it has become a simple cell wall to the egg, and as such takes part in the later process of cleavage.

The zona pellucida persists unchanged as the so-called vitelline membrane of the egg at the time of fertilization and during the early stages of embryonic development.

As in other amphibian eggs, only these two membranes, the zona pellucida and the cell wall formed from the zona radiata, accompany the egg in its escape from the ovary; the process of ovulation involves the rupture of the follicle which remains in the ovary.

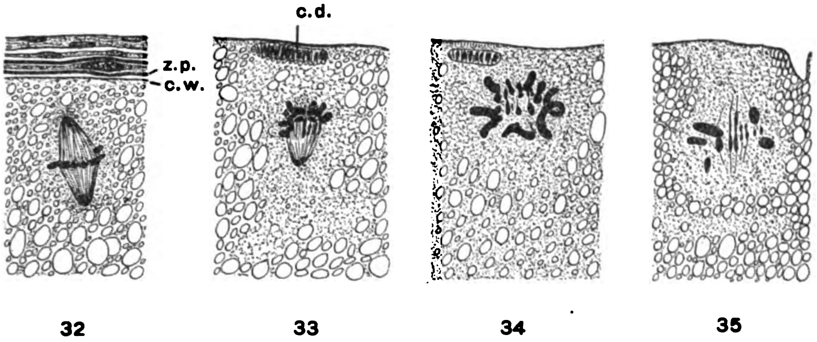
The occurrence of the first polar spindle was studied in two females, (*A*) and (*B*), in which the eggs were distributed from ovary to uterus inclusive. The first polar spindle was found in eggs taken from the following situations: ovary, body cavity, oviduct, and extreme upper part of the uterus; out of a total of twenty-eight eggs studied, the first polar spindle was found in thirteen cases. Five eggs taken from the lower uterus were studied; no first polar spindle was found.

In the case of another female (*C*), in which the eggs were all in the uteri, no first polar spindle was found in three eggs sectioned.

Allowance must be made for the fact that in some cases in which the first polar body is absent it may have been missed on account of imperfections in the series. The results are sufficient to justify the conclusion that the first polar spindle is usually present at the time the egg leaves the ovary and during its passage down the oviduct, and that it disappears about the time the egg reaches the uterus.

The first polar spindle (see figs. 32 to 35) is formed with its long axis either coinciding with the axis of polarity of the egg, or oblique to this axis. The number of chromosomes is probably twelve before any of them have divided. There is an outer ring of six large chromosomes, surrounding a central group of six small chromosomes usually found in a state of division; it is probable that these six small chromosomes are not all of equal size. These size differences of the chromosomes are interesting in the light of well-known recent work indicating individual differences in the chromosomes of many forms.

There is frequently present close to the cell wall overlying the spindle a disc-shaped body with an irregular cross-striated structure, which, from its probable mode of origin, I shall call the 'contact disc' (see figs. 33 and 34). This disc takes the cytoplasmic stain, and seems to be of the same composition as the cell wall. The adjacent cell wall is slightly thickened and sometimes shows a cross-striation, reminding one of the zona radiata (compare the effect on the cell wall of penetration by the spermatozoon, described later). The presence of the contact disc is uniformly accompanied by a deficiency of the spindle, which lacks an aster at the end nearest the disc. In a few cases there seems to be a small amount of sphere substance underlying the contact disc. The inference seems to be that the contact disc is the product of the aster of the first polar spindle modified by contact with the cell wall. The function of the disc, if it have any function, may be to anchor the spindle at the surface during the pulling-apart of the two sets of chromosomes. Unfortunately for this hypothesis the linin threads have not been traced from the chromosomes of the first polar spindle to the contact disc; but since the latter structure is never found except in conjunction with a polar spindle, there is no escape from the conclusion that it is in some way related to it.



Figs. 32–35 Meridional sections showing first polar spindle of Cryptobranchus allegheniensis. Figs. 32 to 34 are from ovarian eggs; fig. 35 is from an egg taken from the lower part of the oviduct. × 340.

Fig. 32 *c. w.*, cell wall, formed from the zona radiata. *z. p.*, zona pellucida.

Fig. 33 *c. d.*, contact disc.

Fig. 34 The section cuts the spindle obliquely and includes all the chromatin except one small chromosome belonging to the central part of the group, which is left in an adjacent section. There are probably six large chromosomes forming a ring, surrounding six small chromosomes in a state of division.

Fig. 35 A considerable part of the chromatin is left in an adjacent section. There are probably six large and six small chromosomes, arranged much as in the preceding figure.

Sections afford no explanation of the faint dark spot or shallow depression noted in surface views of the animal pole after the rupture of the germinal vesicle and before the formation of the second polar spindle. An actual depression overlying the first polar spindle is rarely found in sections; if present in the living egg it must be lost through shrinkage of the egg during the process of preparation for sectioning by the paraffin method.

The yolk granules immediately adjacent to the cytoplasm surrounding the spindle are distinctly larger than at the same level elsewhere; they are doubtless brought from a deeper situation by the migration of the nucleus.

The anaphase of the first polar spindle has not been observed, and the first polar body has been found only in a state of degeneration, in conjunction with the second polar spindle.

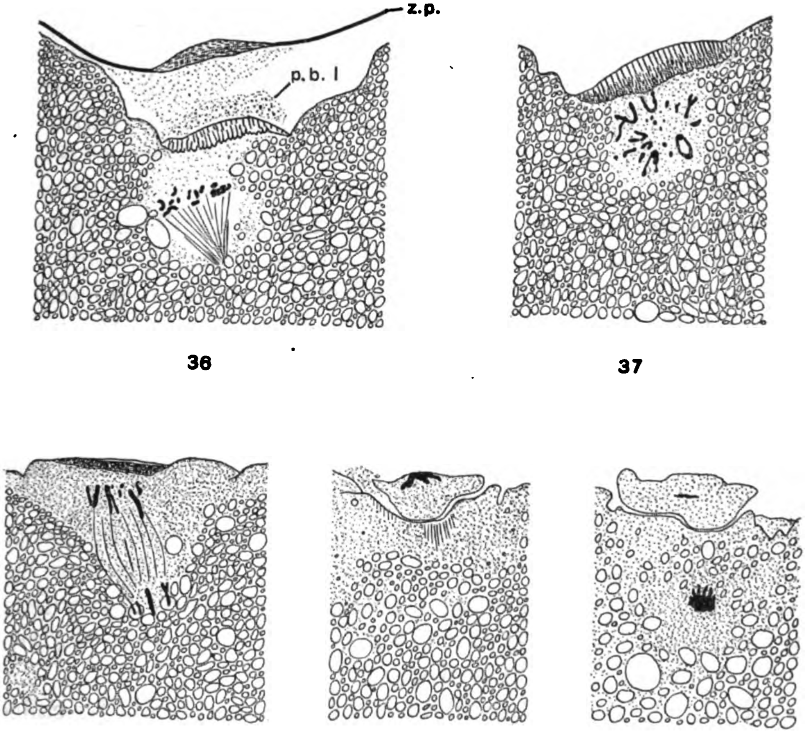
3. The second polar spindle

The second polar spindle (see figs. 36–38) may be distinguished from the first by the smaller amount of chromatin material, and by the fact that a well-defined pit already noted in surface views usually lies above it. This pit sometimes disappears in the late anaphase of the spindle.

The débris of the first polar body is usually found at the bottom or sides of the pit, outside of the cell wall; in some cases fragments of its chromatin are found mingled with the contact disc of the first polar spindle. The chromatin fragments stain but faintly with borax carmine.

The contact disc of the first polar spindle has fused with the thickening of the cuticle which overlies it. In the telophase of the second polar spindle a new contact disc is formed which soon fuses with the old. In some cases linin threads have been clearly traced from chromosomes to the contact disc of the early second cleavage spindle, thereby sustaining the view of the origin of the contact disc set forth in the account of the first cleavage spindle.

No second polar spindle has been found in any eggs of the two females, (*A*) and (*B*), for which the occurrence of the first polar spindle was tabulated. This indicates that the second polar spindle is not formed until after the eggs have been for some time



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Figs. 36 to 40 Meridional sections showing the second polar spindle, and the formation of the second polar body and the egg-nucleus in Cryptobranchus allegheniensis. × 340.

Fig. 36 Section through the second polar spindle of an unfertilized egg taken from the uterus of a ripe female. *z. p.*, zona pellucida; *p. b. I*, débris of the degenerating first polar body.

Fig. 37 Section through the second polar spindle of an egg killed 15 minutes after fertilization. The section lies in the plane of the equator of the spindle.

Fig. 38 Late anaphase of the second polar spindle in an egg killed 2½ hours after fertilization. A considerable part of the chromatin is left in an adjacent section.

Figs. 39 and 40 Two consecutive sections through an egg killed 5 hours after fertilization; the first figure shows the second polar body, the second figure shows in addition the newly-formed egg-nucleus.

in the uterus. In the third female (*C*) considered, in which all the eggs were in the uterus, a second polar spindle was found in one out of the three eggs sectioned. This result is sufficient to show that sometimes, if not always, the second polar spindle is formed while the egg is still in the uterus, previous to fertilization; hence the penetration of the egg by the spermatozoon is not required as a stimulus to the formation of the second polar spindle.

The question arises whether the second polar spindle is normally or ever present after the penetration of the egg by the spermatozoon; in other words, do the processes of maturation and fertilization overlap? We must first take into consideration the possibility that eggs dissected from the uterus of a ripe female for purposes of artificial fertilization may not be quite so far advanced as eggs spawned and fertilized in a natural manner. Fortunately it has been possible to check results obtained through artificial fertilization by comparison with a case in which fertilization occurred in a more natural manner. For the study of fertilization three females, (*C*, *D*, and *E*), were principally used; eggs from the gravid uteri of the first two were artificially fertilized in the usual way; the third female spawned with a ripe male while the two were being carried in a pail of water.

Furthermore we must distinguish between what might be called potential fertilization, the mere contact of the seminal fluid with the gelatinous envelopes of the eggs, and actual fertilization, the penetration of the egg proper by the spermatozoon. While the act of fertilization is not consummated until the fusion of the germ-nuclei, the influence of the spermatozoon is felt in many ways as soon as it enters the egg cytoplasm, so that actual fertilization may be said to begin as soon as the spermatozoon pierces the cell wall of the egg. The time record is almost necessarily reckoned from the moment of mixing of the two sexual elements, or potential fertilization; actual fertilization follows after an interval necessary for the passage of the spermatozoon through the gelatinous envelope, which varies for the individual eggs and especially for eggs of different spawnings fertilized by different males, and which can be determined only by a careful microscopical examination of serial sections of each egg.

Out of twenty-one eggs from three females (*C*, *D* and *E*), preserved at intervals extending from fifteen minutes to two and one-half hours after fertilization, a second polar spindle was found in eighteen cases, and one or more spermatozoa were found in each of eleven eggs. The sections show that the spermatozoon may pierce the cell wall of the egg as early as fifteen minutes after contact with the outer envelopes, though a longer time is usually required.

Making allowance for faults of technique we may say that the second polar spindle is usually and probably always present from the time of fertilization up to two and one-half hours later, reckoned from the moment of mixing the sexual elements; there is no essential difference in this respect between eggs artificially and naturally fertilized.

Only early stages of the second polar spindle are found in eggs up to and including one and one-half hours after fertilization; exclusively anaphase stages are found in eggs taken one and three-quarters to two hours after fertilization; the formation of the second polar body and the egg-nucleus (see figs. 39 and 40) is confined to a period between 4 and 8 hours after fertilization. While a stimulus from the spermatozoon is not required to initiate the formation of the second polar spindle, it is evident that the later stages of this mitosis are passed through only after fertilization; in other words, the processes of maturation and fertilization overlap. Hertwig ('06) makes the general statement that in nature the time of fertilization of the amphibian egg falls between the formation of the first and second polar spindles.

4. *The organization of the egg immediately before fertilization*

At the time of spawning the egg is surrounded by the unchanged zona pellucida or vitelline membrane; within this is a thin cell wall, the transformed zona radiata, which is organically connected with the egg.

There are few changes in the general appearance of the blastodisc since the condition described shortly after the rupture of the germinal vesicle (see fig. 31). There is a more intimate incorporation of the materials of the germinal vesicle into the substance of the blastodisc; shreds of non-formative material, such as fragments of the nuclear wall and the fibrous material of the germinal vesicle, are each surrounded by a closely adherent film of cytoplasm and are being absorbed. In eggs ready for fertilization the second polar spindle is sometimes, though perhaps not always, fully formed; it lies beneath a sharply-defined pit at the bottom of which may be found the débris of the first polar body.

The peripheral zone of fine yolk particles in the vegetal hemisphere remains as described in the late ovarian egg.

C. FERTILIZATION

1. *The history of the egg-nucleus*

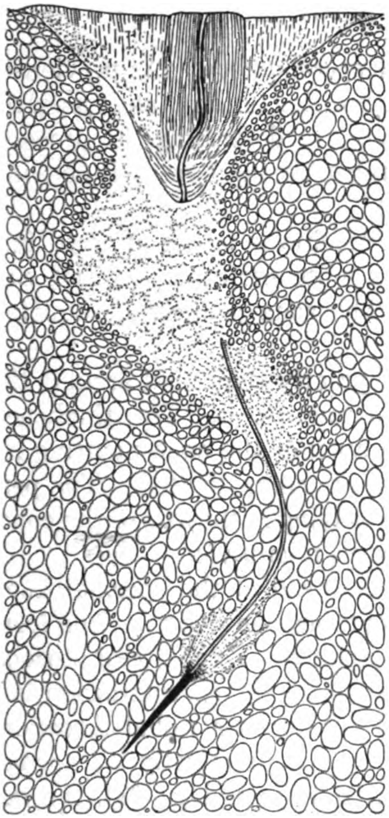
The formation of the egg-nucleus is shown in figures 38 to 40; the process is usually complete about five hours after fertilization. About ten and one-half hours after fertilization (see figs 47 and 48) the egg-nucleus has increased in size and sunk into the blastodisc to a point one-third as far from the surface as the position later occupied by the copulation-nucleus (see fig. 52). A yolk-free region, partly filled with cytoplasm, extends from the egg-nucleus for a short distance toward the surface, indicating the path of migration (fig. 48). At this time the egg-nucleus stains but faintly.

Figs. 41 to 43 Vertical sections of eggs of Cryptobranchus allegheniensis, showing penetration of the egg by the spermatozoon. × 240.

Fig. 41 From an egg killed 2½ hours after fertilization. This figure is a reconstruction from two adjacent sections: the upper half of the figure is drawn from one section, the lower half from the other. The spermatozoon shown in the figure has entered the egg about 50° from the animal pole where the second polar spindle, shown in fig. 38, is in the late anaphase stage. Another spermatozoon in the same condition as the one figured has entered the opposite side of the egg a little below the equator.

Fig. 42 From an egg killed 3 hours after fertilization. The spermatozoon figured has entered the egg a little above the equator. This egg contains in all ten spermatozoa.

Fig. 43 From an egg killed 5 hours after fertilization. The arrow indicates the direction of the path of the spermatozoon which has entered the egg about 30° from the animal pole. The distance from the surface of the egg to the head of the spermatozoon is about one and one half times as great as in the preceding figures. The head of the spermatozoon is shown entire in this section; the tail persists in a somewhat abbreviated condition, but is not shown in the section figured. This egg contains another spermatozoon in the same condition.



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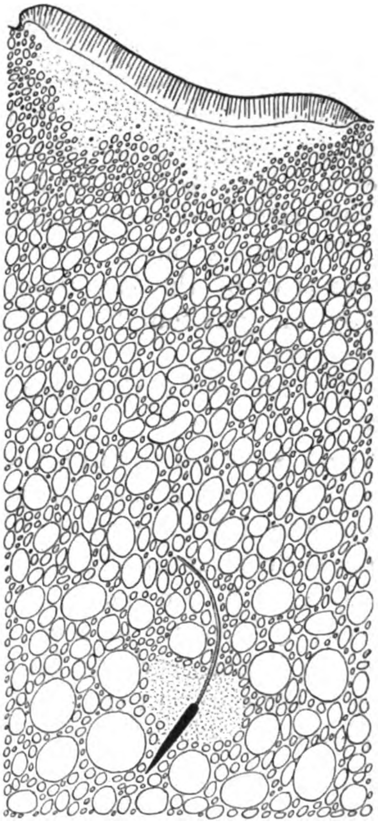

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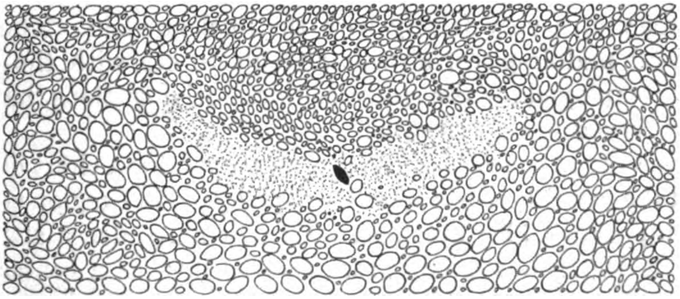

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2. History of the sperm-nucleus

(*a*). *Penetration of the egg by the spermatozoon.* As previously noted, spermatozoa may be found entering an egg taken as early as fifteen minutes after fertilization. In describing the process, we may best begin with an egg taken about two and one-half hours after fertilization (see fig. 41).

The zona pellucida or vitelline membrane is not affected further than by the formation of a minute perforation which can only rarely be found in sections. The zona pellucida is omitted in the figures.

The cell wall of the egg becomes greatly thickened around the perforation made in it by the spermatozoon. The thickened region is conical in form, with the apex of the cone pointing inward; its outer and central portions are cross-striated. The perforation persists as a conspicuous pore lying in the axis of the cone. The entire structure greatly resembles a micropyle.

Beneath this pseudo-micropyle the path of the spermatozoon is clearly indicated by a yolk-free cytoplasmic region. The form of this region, and the attitude assumed by the spermatozoon itself, indicate that the course pursued by the spermatozoon is a spiral one, with the axis of the spiral lying in a radial direction.

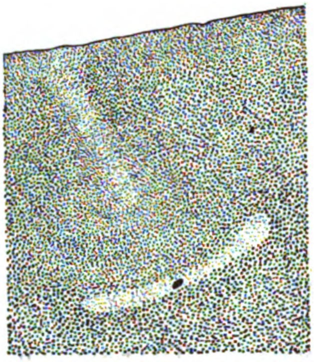
The spermatozoon at this time retains practically its normal form. As in Axolotl (Fick, '93) and Bufo (King, '01), the tail is not left behind at the surface; in Cryptobranchus it continues to serve as an efficient organ of propulsion. The undulating membrane persists, though it is not shown in the figure. The head at this time stains very faintly with the nuclear stain. The acrosome and middle-piece, always difficult to see with the magnification employed for the study of thick serial sections, have not been observed in this situation.

Surrounding the shaft of the spermatozoon for a short distance behind the head there is a spindle-shaped yolk-free region containing cytoplasm. This cytoplasm is particularly dense about the region of the middle-piece; from this locality as a center cytoplasmic strands, resembling linin threads, but finely granular, radiate in all directions, but those extending backward are more prominent. This phenomenon is much more marked in some other cases than in the one figured.

In eggs taken about three hours after fertilization (see fig. 42), the thickening of the cell wall has flattened to the form of a disc; it is strongly striated, recalling the zona radiata from which it takes its ultimate origin. The perforation made by the spermatozoon has disappeared. The cytoplasmic path of the spermatozoon has become filled with yolk, except for a broad shallow region underlying the thickening of the cell wall. The head of the spermatozoon has become shorter and thicker, and takes brilliantly the nuclear stain; the tail has become slightly shorter, perhaps by the degeneration of the posterior portion. The radiations of cytoplasm proceeding from the region of the middle-piece have disappeared, but in the same locality there is a somewhat larger spherical region of uniformly distributed yolk-free cytoplasm.

Five hours after fertilization (see fig. 43), the spermatozoon has penetrated only a little deeper into the egg. The thickening of the cell wall of the egg at the place of entrance of the spermatozoon has disappeared, but its site is marked by convolutions in the cell wall. The protoplasmic path leading from the surface of the egg to the spermatozoon has almost entirely disappeared, but traces of it persist at intervals along the route. The head of the spermatozoon is spindle-shaped and much shorter and thicker than before; the tail persists, but is somewhat abbreviated. The circular area of cytoplasm surrounding the head of the spermatozoon has expanded to form a large crescent, whose horns extend nearly at right angles to the path of the spermatozoon. The yolk granules underlying the crescent are decidedly coarser than those above it. This suggests a correlation of the internal structure with the 'sperm area' seen from the surface: the horns of the crescent produced would meet the margin of the sperm area (compare figs. 9 to 11 with figs 43 to 45).

Seven and one-half hours after fertilization (see fig. 44) the protoplasmic path is marked only by a region of sparsely distributed yolk granules extending from the surface for about two-thirds of the distance to the spermatozoon. The yolk granules are particularly dense in the region immediately above the crescent, and



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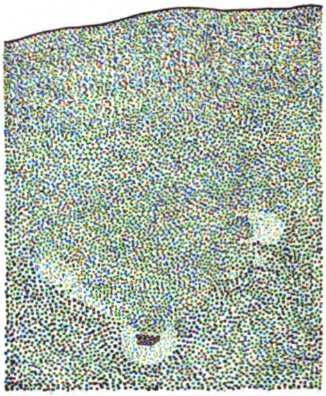

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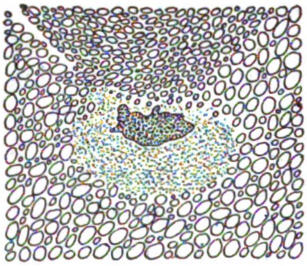

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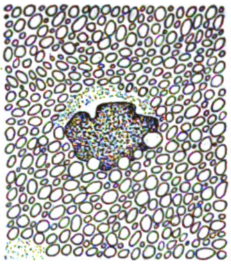

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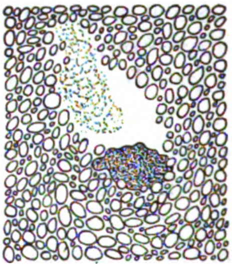

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Fig. 44 Vertical section through an egg of Cryptobranchus alleghenienisis killed 7½ hours after fertilization, showing a late stage in the penetration by the spermatozoon. × 80. This spermatozoon has entered the egg about 30° from the animal pole, and its path inclines toward the axis of polarity of the egg. The head of the spermatozoon is shown entire; the tail persists in an abbreviated and perhaps fragmented condition, but does not appear in the section figured. An aster is present in an adjacent section at a little higher level than the sperm head. Another spermatozoon in the same general condition is found in the same egg.

Fig. 45 Vertical section through an egg killed 10½ hours after fertilization, showing the sperm-nucleus. × 80. The spermatozoon has entered the egg about 25° from the animal pole. Fragments of the tail of the spermatozoon are to be found in the vicinity of the sperm-nucleus, but are not shown in this section.

Fig. 46 A portion of fig. 45 enlarged to show the sperm-nucleus. × 240.

Figs. 47 and 48. Two consecutive meridional sections through an egg of Cryptobranchus alleghenienisis, killed 10½ hours after fertilization, showing the egg-nucleus. This nucleus is situated about one-third as far from the surface as the copulation-nucleus shown in fig. 52. × 240.

are here finer than elsewhere at the same level. The crescent has become larger, and thicker at the ends than in the middle. The head of the spermatozoon is shortened to the form of a thick spindle and stains deeply; the tail persists in an abbreviated condition. In the case of the spermatozoon shown in figure 44, an aster is found at a slightly higher level than the sperm head and a little nearer to the egg-nucleus. A study of the protoplasmic paths of the aster and the sperm-head shows that they have separated at a point midway in the path of the latter.

Ten and one-half hours after fertilization (see figs. 45 and 46) the spermatozoon has become transformed into the sperm-nucleus, which is amoeboid in form; the tail of the spermatozoon is represented only by fragments. At this time the sperm-nucleus lies about half as far from the surface as the copulation-nucleus shown in fig. 52. Immediately beneath the sperm-nucleus lies a considerable mass of cytoplasm, perhaps formed at the expense of the crescent which is dwindling except at the extreme ends. The remains of the crescent, and the characteristic appearance of the surrounding yolk, enable one readily to distinguish the sperm-nucleus from the egg-nucleus. The sperm-nucleus is smaller than the egg-nucleus, and like the latter does not stain deeply at this time.

The cytoplasmic changes in the egg caused by the invasion of the spermatozoon may be tentatively interpreted as follows: Under the influence of the centrosome, whose seat appears in this case to be in the middle-piece, egg-cytoplasm collects about the neck of the spermatozoon. Here the centrosphere and eventually the entire aster is formed. As the spermatozoon invades the deeper region of coarser yolk particles, the resistance offered to the progress of the accompanying mass of cytoplasm causes it to flatten out like a bullet fired against a wall, assuming a form crescent-shaped in section. Presently the spermatozoon, during its transformation into the sperm-nucleus, comes almost to a full stop, allowing the mass of cytoplasm again to assume a spherical form.

Numerous observers have described in both invertebrates and vertebrates a rotation of the sperm head after it enters the egg, whereby the middle-piece is brought into position to precede during the further process of migration. Fick ('93) has described this process in Axolotl, and Dean ('06) has noted it in Chimaera. King ('01) found no indication of a rotation of the sperm head in Bufo; possibly this condition is correlated with the fact that in Bufo the centrosome appears to be located, not in the middle-piece, but in the head of the spermatozoon. In Cryptobranchus rotation of the sperm head apparently takes place at a rather late stage in the process of transformation into the sperm-nucleus. In the stages shown in figures 43 and 44, the greatly shortened sperm head is usually placed with its long axis oblique or at right angles to its former path, so that one end points toward the egg nucleus. But in these stages it has not been possible to trace any connection between the tail of the spermatozoon and its head, and since the aster has already separated from the sperm head, in no case can it be stated which end of the sperm head is the one pointed toward the egg-nucleus.

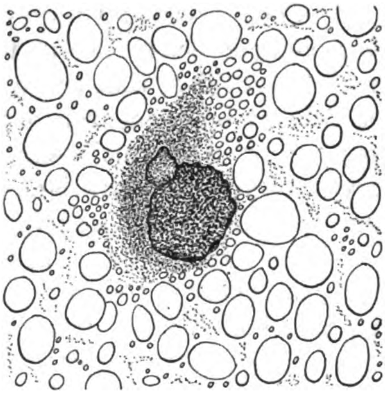
The spermatozoon ordinarily enters the blastodisc in a more or less centripetal direction, and continues in this direction for a considerable distance; sometimes its path inclines almost from the beginning in an oblique direction toward the point of future union with the egg-nucleus. In either case the axis of the spiral path is ordinarily straight up to the time of the transformation of the head of the spermatozoon into the sperm nucleus; the later course of migration has not been followed. In an egg preserved an hour and three-quarters after fertilization, a spermatozoon, which had entered the blastodisc unusually near the animal pole, described a path which proceeded in a centripetal direction only a very short distance, then curved sharply in a direction parallel to the surface, toward the second polar spindle which was in the late anaphase condition. The form of the spermatozoon remained unaltered, and rotation of the sperm head had not commenced. This case is instructive in showing that the factors tending to bring the germ-nuclei together are active at a very early stage of fertilization: the egg-nucleus was not fully formed, and the spermatozoon had not begun its process of transformation into the sperm-nucleus. Moreover it is evident that in this case at least the 'copulation path' (see Hertwig, '06, p. 529) is not dependent upon the rotation of the sperm head.

(*b*). *Polyspermy, and the fate of the supernumerary spermatozoa.* Brief data regarding the occurrence of polyspermy have already been given. It is possible that the method of artificial fertilization increases the number of spermatozoa entering the egg; but in nature the eggs are fertilized in a confined space, and I see no reason to doubt that polyspermy is a common occurrence under natural as well as artificial conditions. It is evident that we have here to deal with physiological, not induced or accidental polyspermy (see Brachet, '10), for the eggs develop in a normal manner.

While the distribution of spermatozoa entering the egg is largely if not entirely a matter of chance, the location in which a spermatozoon finds itself has much to do with its ultimate fate. Spermatozoa entering the lower hemisphere, especially in the region of the vegetal pole, never penetrate far, and since they are found in this hemisphere only during the first few hours after fertilization, must quickly degenerate. In the urodele Hynobius, Kunitomo ('10) found that a spermatozoon entering at the vegetal pole sometimes succeeds in reaching the egg-nucleus; but the careful study of many eggs has convinced me that this never occurs in the heavily yolk-laden and strongly telolecithal egg of Cryptobranchus.

Only in the blastodisc have spermatozoa been found in the stage characterized by the presence of the cytoplasmic crescent (figs. 43 and 44). Obviously, the conditions elsewhere are unfavorable for the formation of any considerable mass of cytoplasm about the spermatozoon. In the stage with a well-formed cytoplasmic crescent, not more than two spermatozoa have been found in a single egg. No accessory spermatozoa have been found in any situation after the formation of a sperm-nucleus. The supernumerary spermatozoa thus have but a transient existence, and the only advantage resulting from polyspermy is doubtless that, in an egg so large, penetration by several spermatozoa is of value in insuring fertilization.

The literature on the occurrence of polyspermy in the amphibian egg has recently been reviewed by Kunitomo ('10). As noted in a previous paper (Smith, '11), I have found polyspermy occurring in eggs of Amblystoma tigrinum fertilized under natural conditions; the material secured for the further investigation of this subject has not yet been worked up. Polyspermy seems to be characteristic of heavily yolk-laden eggs lacking a preformed micropyle.



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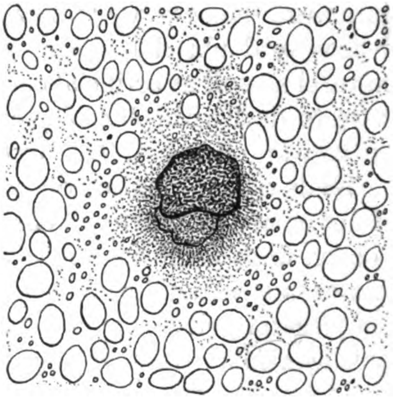

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Fig. 49 Meridional section through an egg of Cryptobranchus allegheniensis, killed 12 hours after fertilization, showing fusion of germ-nuclei. The sperm-nucleus is probably the smaller one. × 240.

Fig. 50 Meridional section showing fusion of germ-nuclei in another egg killed 12 hours after fertilization. The sperm-nucleus is probably the lower and smaller one. × 240. For the position of this copulation-nucleus in the blastodisc see fig. 52 which is drawn from the same section.

3. Union of the germ-nuclei, and formation of the first cleavage spindle

In two eggs killed twelve hours after fertilization, the germ-nuclei have been found in the process of uniting (figs. 49 and 50); in these two cases the copulation-nuclei are at approximately the same distance from the surface (see fig. 52), quite deeply situated in the blastodisc and a little to one side of the axis of polarity. The smaller germ-nucleus is probably the sperm-nucleus. The egg-nucleus stains brilliantly with borax-carmine; the sperm-nucleus takes the stain less deeply. The sperm-nucleus especially is surrounded with dense cytoplasm; in one case (fig. 50) this exhibits a tendency toward radial striation and probably represents the aster.

The study of the paths of migration of the germ-nuclei and the copulation-nucleus is not quite complete, but indicates that the germ-nuclei come together at a higher level than that occupied by the copulation-nuclei shown in the figures.

The first segmentation nucleus in a resting condition has been found in an egg killed eighteen hours after fertilization; the first cleavage spindle has been found in an egg killed seventeen hours after fertilization.

4. *Changes in the blastodisc*

In eggs taken from fifteen minutes to ten and one-half hours after fertilization, cytoplasm is accumulating in irregular patches underlying the animal pole (fig. 51). During this period, practically all traces of the débris of the germinal vesicle disappear. In places, the surface of the blastodisc is sometimes very irregular, almost villous; this may be due to injuries resulting from the actual or attempted entrance of spermatozoa.

In eggs taken from twelve to eighteen hours after fertilization (copulation nucleus to first cleavage spindle) the cytoplasm is gathering in a broken layer close to the surface of the blastodisc. The beginning of this process is shown in figure 52. In Hynobius, Kunitomo ('10) has noted a somewhat similar condition. During the latter part of the period considered the layer of cytoplasm becomes much thicker than is shown in the figure, but retains its segmented character.

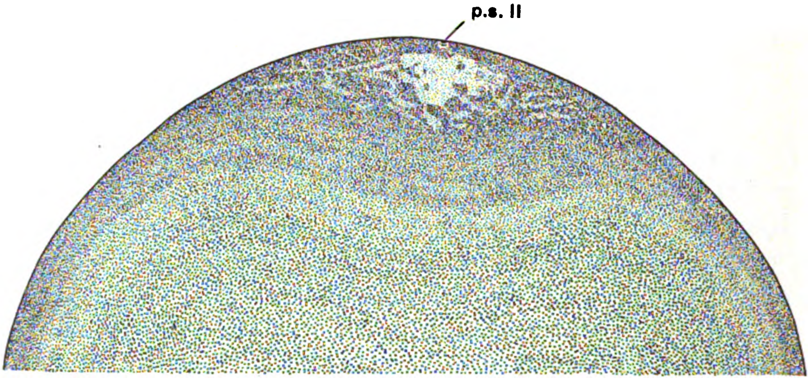
During the first two hours after fertilization there is a marked increase in the thickness and extent of the blastodisc as a whole (see especially fig. 51). Evidently the greater part of this change takes place before the egg has become oriented with the animal pole uppermost, hence it is independent of any possible sorting effect of gravity acting on the materials of the egg.

No marked changes have occurred in the lower hemisphere since the egg left the ovary.

The later changes in the blastodisc lead up to first cleavage and will be considered in that connection.

D. SUMMARY

The follicular layer proper of the ovarian egg of Cryptobranchus is formed from some of the deeper non-germinal cells of the ovarian wall which resemble the epithelial cells of the outer and inner limiting membranes. The follicular membrane proper completely surrounds the egg and is suspended in a two-layered flask-shaped sac which projects from the inner surface of the wall of the ovary



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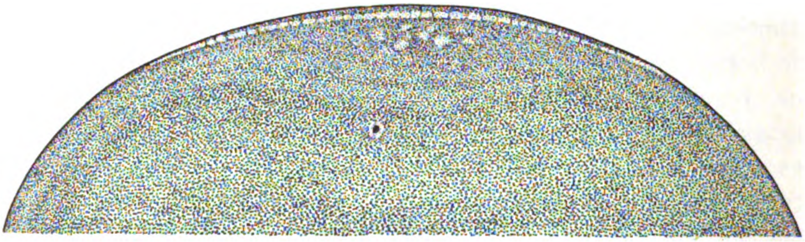

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Fig. 51 Meridional section through an egg of Cryptobranchus allegheniensis, killed 1¾ hours after fertilization, showing the condition of the blastodisc. The irregular faintly stippled areas near the animal pole contain yolk-free cytoplasm. × 18. *p. s. II*, second polar spindle.

Fig. 52 Meridional section through an egg of Cryptobranchus allegheniensis, killed 12 hours after fertilization, showing condition of the blastodisc and position of the copulation-nucleus. Yolk-free cytoplasm is segregated in a broken layer near the surface of the blastodisc. The copulation-nucleus is shown a little to the left of the center of the figure. × 18.

into the central cavity; in a broad sense, the entire three-layered structure may be called the follicle.

The zona radiata is formed from the peripheral substance of the egg proper; at the time of the rupture of the germinal vesicle it becomes transformed into a simple cell wall, in organic connection with the egg.

The zona pellucida is formed as a secretory product of the follicular layer proper; it persists unchanged as the 'vitelline membrane' of the embryo.

The earliest observed phenomena which may perhaps indicate polarity occur in the ovarian eggs of young females of a body length of 26 to 30 cm., as a shifting of the region of most abundant vitelline bodies from the future vegetal to the future animal hemisphere. In the ovarian eggs of young females of a body length of 35 cm there is a concentration of nucleoli on the side of the germinal vesicle toward the future animal pole; this may perhaps afford a second indication of polarity.

Yolk-formation begins in the most advanced ovocytes of young females with a body length of 35 cm.; the yolk is first laid down in concentric zones. With respect to the position of the germinal vesicle, the distribution of cytoplasm, and the size of the yolk particles in the different zones, the egg exhibits radial symmetry until after it is nearly or quite filled with yolk.

About the time when the egg becomes completely filled with yolk, the germinal vesicle migrates from its central position toward a point on the surface which is thus defined as the animal pole. Coincident with the migration of the germinal vesicle, axial differentiation of the cytoplasmic and yolk contents of the egg lead to the formation of a germinal disc in the region of the animal pole.

In general the animal pole of the egg lies within the stalk of the follicle and toward the periphery of the ovary.

In the late ovarian egg a structure called the yolk cup is interpreted as the physiological equivalent of the concentric layers of dense fine yolk found in the eggs of birds and various other vertebrates.

Shortly before maturation the germinal disc is temporarily differentiated into two layers: a thin peripheral layer of yolk-free cytoplasm, and underlying this a thicker layer of especially fine yolk particles rich in cytoplasm. Both layers are continuous with much thinner layers of the same character surrounding the remainder of the egg.

In the ovocyte ready for maturation, the germinal vesicle lies close to the surface at the animal pole, and is surrounded by the germinal disc. A mass of cytoplasm has accumulated beneath the germinal vesicle during the later stages of its migration. The arrangement of materials is now quite strongly telolecithal.

Shortly before the rupture of its wall, the germinal vesicle appears at the very surface at the animal pole. The rupture of the germinal vesicle takes place just before the egg leaves the ovary; the cytoplasmic and yolk layers of the blastodisc now mingle, and the materials of the germinal vesicle, together with the cytoplasm brought with it from the interior of the egg, are incorporated into the blastodisc.

Absorption of degenerating ovocytes is accomplished by means of the follicle cells, which reverse their usual rôle as nurse cells of the egg, and function as phagocytes.

The first polar spindle is formed about the time the egg leaves the ovary, and disappears about the time the egg enters the uterus. There are marked size differences in the chromosomes.

The second polar spindle is formed shortly after the egg enters the uterus; it lies beneath a deep pit readily visible from the surface.

The penetration of the egg by the spermatozoon is not required as a stimulus to the formation of the second polar spindle.

The late stages of the second maturation division, culminating in the formation of the second polar body and the egg-nucleus, are passed through only after the spermatozoon has entered the egg; in other words, the processes of maturation and fertilization overlap.

A structure resembling a micropyle is formed in the cell wall of the egg around the perforation made by the entrance of the spermatozoon. The influence of the entering spermatozoon upon the egg is shown by characteristic changes in the distribution of the yolk and cytoplasm.

Physiological polyspermy is of normal occurrence. The supernumerary spermatozoa lead but a transient existence.

Union of the germ-nuclei takes place at a point deeply situated near the center of the blastodisc, and is followed by the segregation of masses of cytoplasm forming a broken layer near its surface.

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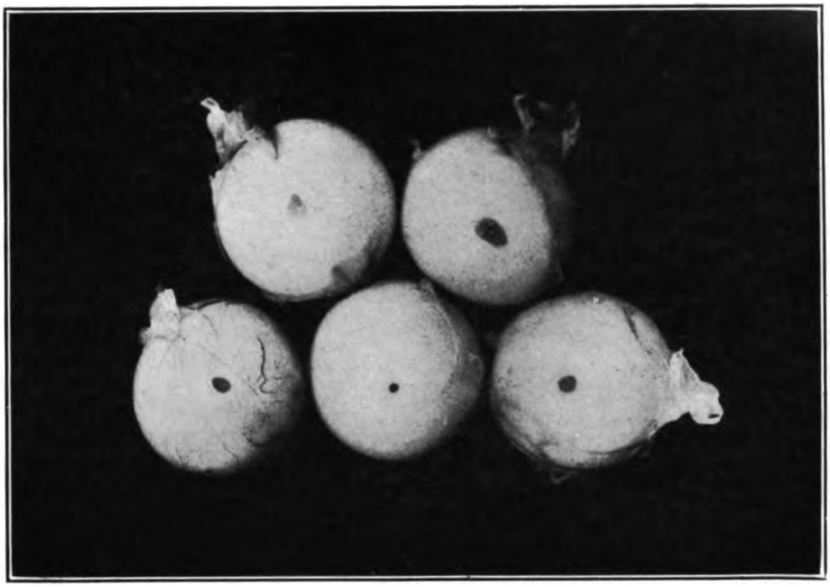
PLATE 1

EXPLANATION OF FIGURES

53 Cryptobranchus allegheniensis. Living eggs dissected from the ovary, showing the germinal vesicle at the surface. The small spots within the germinal vesicle are probably nucleoli. Ovarian membranes containing blood-vessels wholly or partially cover the eggs. × 4.

54 Cryptobranchus allegheniensis. Unfertilized eggs with their gelatinous envelopes, after two days' immersion in water. Natural size.



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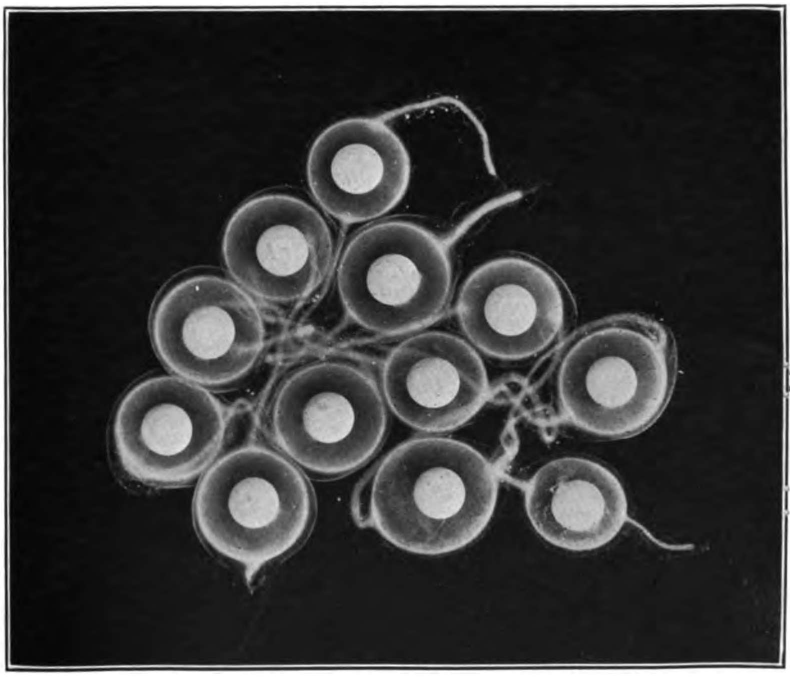

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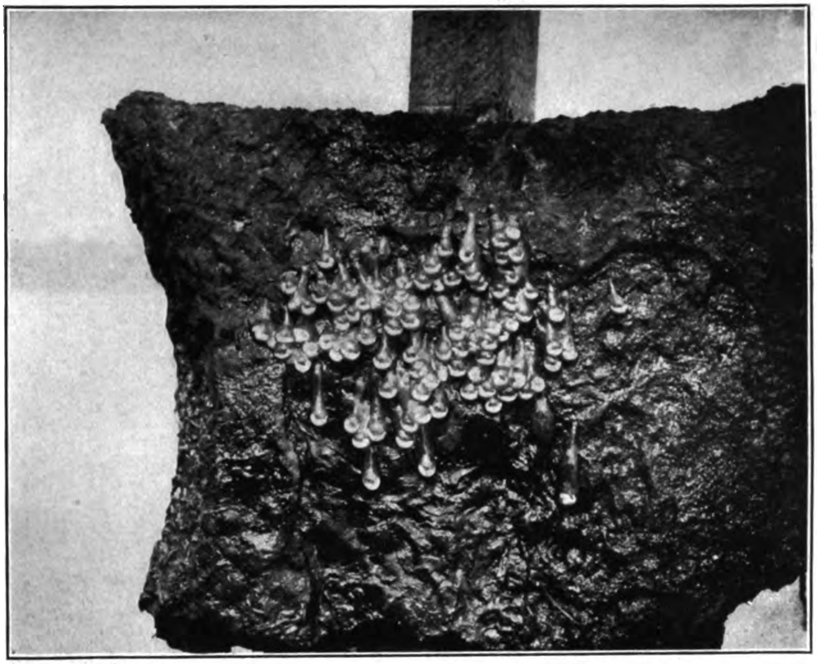
PLATE 2

EXPLANATION OF FIGURES

55 Necturus maculosus. 'Nest' of eggs of Necturus. The stone to which the eggs are attached has been removed from the water and set on edge on the wharf; it is about 16 inches in diameter. The embryos are in an advanced stage of development.

56 Necturus maculosus. Eggs and envelopes shown in their natural position in the water, attached to a piece of board; natural size. From a drawing by Professor Bashford Dean.



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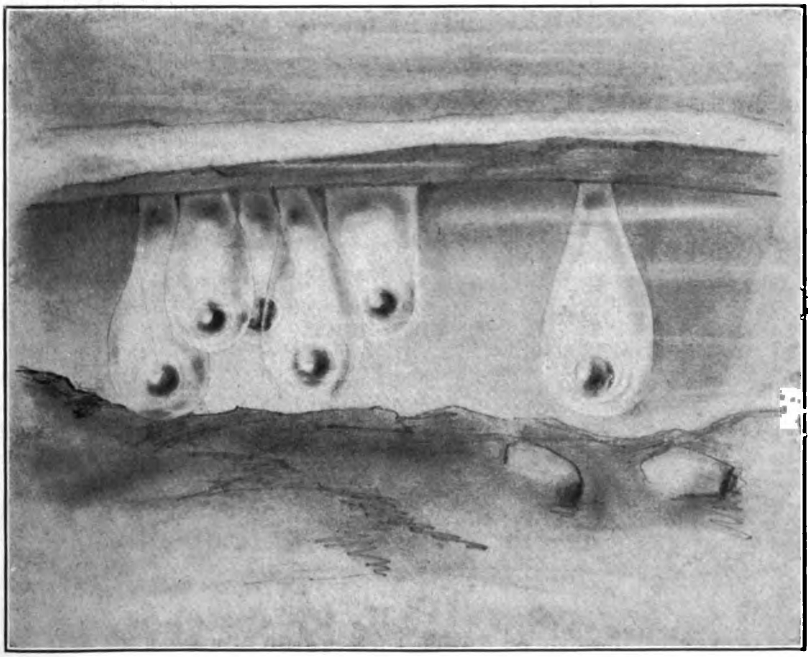

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UBM

VITA

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The Embryology of Cryptobranchus Allegheniensis

Including Comparisons with Some Other Vertebrates

Part II

General Embryonic and Larval Development, with Special Reference to External Features

BY

BERTRAM GARNER SMITH

Submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy, in the Faculty of Pure Science, Columbia University

Reprinted from the Journal of Morphology, vol. 23, no. 3, September, 1912

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II. GENERAL EMBRYONIC AND LARVAL DEVELOPMENT, WITH SPECIAL REFERENCE TO EXTERNAL FEATURES[1]

BERTRAM G. SMITH

From the Zoological Laboratory of Columbia University

TWO HUNDRED AND TWENTY-THREE FIGURES (EIGHT PLATES)

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VI. INTRODUCTION

The present contribution is one of a series of papers on the embryology of Cryptobranchus, of which Part I, dealing with the breeding habits, ovogenesis, maturation and fertilization, has already been published (Smith '12). In the preparation of this paper I am greatly indebted to Prof. Bashford Dean, under whose guidance it has been brought to completion.

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[1] Part I was published in the Journal of Morphology, vol. 23, no. 1, March, 1912.

During the past seven years the collection of an abundance of material has enabled me to preserve an ample supply in every stage of development. At least fifteen thousand embryos have been secured from nests, and nearly as many more have been obtained by artificial fertilization.

For convenience in description, the embryonic and larval history has been divided into stages, based chiefly on external characters. In making the division, the usual difficulty has been encountered, that the rate of development of each structure varies more or less in different embryos. In determining what shall constitute the interval between stages, the guiding principle has been to establish stages only so far apart that individual variations in the rate of development of the most important characteristics selected as criteria for classification shall not overlap. For purposes of more intensive study, each stage may be divided into tenths; this device is useful in following any single character or set of characters. Since development is a continuous process, the importance of studying a close series cannot be too strongly emphasized.

As an aid to obtaining the exact sequence of events, stress has been laid on the study of a series of stages preserved at short intervals, from a single lot of eggs fertilized at the same time. Every period of the embryonic development has been covered repeatedly in this way, an entire spawning of eggs being sometimes used in the study of three or four stages as distinguished in this account. Thus not only a close series, but a large number of embryos in each stage, representing several different spawnings, have been studied, so that the typical course of development could readily be distinguished from variations or abnormalities.

Moreover, the entire embryonic and larval history has been carefully followed in living material, repeatedly and for the most part with embryos freshly collected. The study of living material is of especial importance in the late cleavage and gastrula stages of Cryptobranchus; for in these stages the translucent condition of parts of the unpigmented embryo enables one to gain a fair idea of what is going on inside. The ciliation of the ectoderm of the late embryo, and some features of the circulatory system, are best studied in living material.

An accurate and complete time record (Section X) of the course of development has been obtained by comparison of many different records of material kept alive during long periods of time; these results were checked by observing the rate of development of material freshly collected. One lot of embryos, collected in the fall of 1906 at the time of the closure of the neural folds, were kept alive in the Zoological Laboratory of the University of Michigan throughout the entire larval period, and their metamorphosis was observed at the end of the second year after fertilization. Specimens were preserved at intervals; shortly after metamorphosis the half-dozen remaining individuals died from causes unknown. Another lot of embryos collected in an advanced gastrula stage in the fall of 1910, were kept alive and in good condition in the Zoological Laboratory of the University of Wisconsin until May, 1911, when the last ones were preserved.

The study of external and internal structure has gone hand in hand, except for the post-gastrula stages; here, doubtful points in the interpretation of the external structure have in most cases been investigated by reference to serial sections.

In preparing the illustrations, composite or ideal figures have been avoided. Each drawing, unless otherwise specifically stated, is a faithful representation of an individual embryo; a sufficient variety of figures has been given to illustrate the most important deviations from the condition regarded as typical. All the drawings are the work of the author except figures 268 to 276 which were drawn by Prof. Bashford Dean and with his generous permission are here published for the first time; figure 203 which was kindly contributed by Dr. L. Hussakoff of the American Museum of Natural History; and figures 277 to 279 which were drawn by Miss Hedge of Columbia University.

The histological technique employed has already been given in Part I; it remains to record the methods used in photography. For embryonic stages, fixation in Solution B (see Part I) followed by preservation in formalin, gives the best photographic results. While being photographed, the objects were immersed in water or formalin. Living larvae were anesthetized with chloretone. In all cases the exposure was made by daylight.

All the photographs with a magnification of × 4 were made with a Bausch and Lomb Zeiss Tessar 72 mm. lens, fitted to a long bellows Pony Premo No. 6 camera. The camera was fastened in an erect position by means of an improvised wooden frame. Figures 262, 264, 265 and 266 were taken with a Zeiss Unar Lens; figures 263 and 267 with a Zeiss Apochromatic Planar. Seed's Non-halation plates were used throughout; they were developed with Adurol.

All the negatives are the work of the author except figure 266 which was made by Miss Frances J. Dunbar of the University of Michigan.

The photographs are untouched, except in a very few cases for the purpose of correcting slight defects in the negatives.

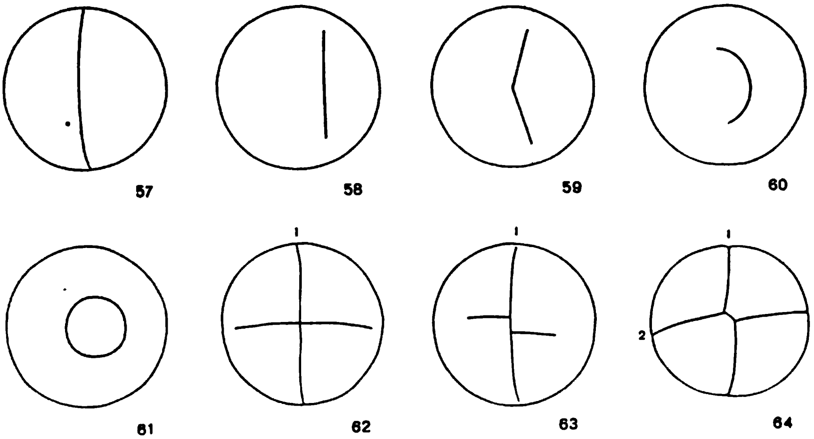
VII. CLEAVAGE

A. Description of cleavage by stages

Stage 1: (figs. 57 to 61 and 204). This stage is characterized by the presence of the first cleavage furrow only. The germinal area reaches nearly to the equator.

In artifically fertilized eggs the first cleavage furrow ordinarily appears about twenty-four hours after fertilization; the time may vary from eighteen to twenty-eight hours. The furrow begins as a pit, usually at the animal pole; it lengthens rapidly at first, then more slowly as it invades the regions of the egg more heavily laden with yolk. After the first cleavage furrow is well established, it becomes narrow in its middle portion, while still broad at the ends. The first cleavage furrow of the typical form becomes superficially complete in Stage 3 (third cleavage).

In the typical condition, the first cleavage furrow passes through the animal pole, and the division is equal (figs. 57 and 204). Variations from this condition occur. To test the amount of variation, sixty eggs from a single spawning were examined in the first cleavage stage. In forty-eight cases the condition was of the typical character described above. In seven cases the first cleavage furrow was straight, but the cleavage unequal; figure 58 represents the extreme of this condition. In three cases, the first cleavage furrow passed through the animal pole, but its halves met at this pole to form an obtuse angle (fig. 59).



Figs. 57 to 64 Types of first and second cleavage of Cryptobranchus allegheniensis. × 3½.

Fig. 57 The first cleavage furrow extends just to the equator, a little further than is usual before the appearance of the second furrow.

Fig. 62 The first cleavage furrow extends a little below the equator.

Fig. 64 The first cleavage furrow extends a little below the equator; the second furrow extends just to the equator.

In two cases the first cleavage furrow formed a semicircle about the animal pole (fig. 60). In two cases from different spawnings, neither of which furnished the material for the above data, circular first cleavage (fig. 61) was found; in each case the animal pole was excentrically situated within the area bounded by the cleavage furrow.

Goodale ('11) reports a case of circular first cleavage in Spelerpes; the egg gave rise to a normal embryo. According to Eycleshymer ('04), in Necturus the cytoplasm is always unequally divided by the first cleavage, giving rise to blastomeres which in many cases are decidedly unequal.

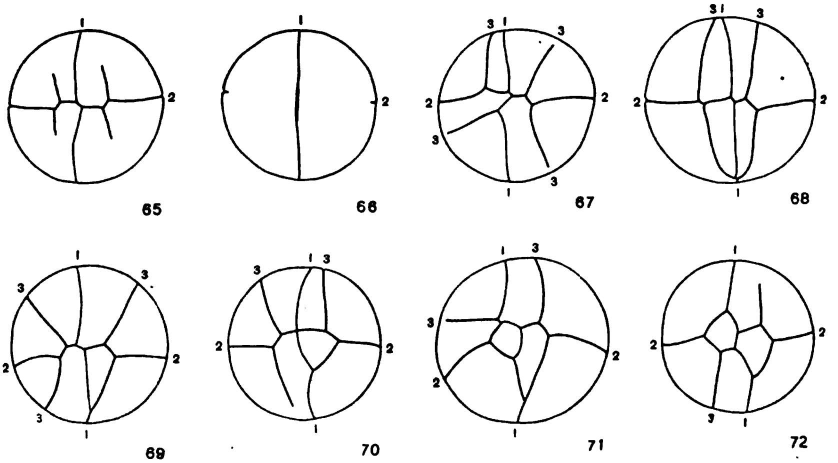
Stage 2: (figs. 62 to 64 and 205). The second cleavage furrow makes its appearance about six hours after the first, which by this time extends nearly to the equator of the egg. In the stage represented in figure 205, the first cleavage furrow has just reached the equator. The second cleavage furrow usually becomes superficially complete in Stage 4 (fourth cleavage), quite uniformly meeting the first cleavage furrow at right angles at the vegetal pole.

The earliest indication of the second cleavage furrow is usually a roughness in the region of the animal pole where the second groove is to intersect the first. The occurrence of 'Faltenkranzen'—a quivering of the surface with the formation of fine radiating or parallel wrinkles, which extend outward from the cleavage furrow—is quite marked at the time of the initiation of the second cleavage furrow. The cause of the formation of similar wrinkles in the frog's egg has been investigated by Charles B. Wilson ('96). For some time after its first appearance the second furrow is much broader, though of course shallower, than the first.

The second cleavage furrows usually depart from the same point as the first furrow, and proceed vertically, forming a single straight line at right angles to the first furrow (figs. 62 and 205). Occasionally the points of departure of the second furrows do not coincide, as shown in figures 63 and 64. The condition shown in figure 63 is rarely observed, and is transitional to that shown in figure 64, which is quite frequent. That portion of the first cleavage furrow lying between the points of departure of the second furrows may be called the polar furrow. As shown by the individual histories of a large number of eggs, in all cases in which a polar furrow is present the points of departure of the second cleavage furrows are separate from the beginning; the polar furrow at first exists as a part of the straight first cleavage furrow, but later becomes oblique through the shifting of cells. In no case in which the second cleavage furrows have their origin from the same point in the first cleavage furrow, has there ever been observed any shifting of cells during this or the following stage, of such a nature as to produce a polar furrow.

Comparison with the second cleavage of Necturus as figured by Eycleshymer ('04), Eycleshymer and Wilson ('10), and in some unpublished drawings of the living egg by Prof. Bashford Dean, leads to the conclusion that in Necturus there is much greater irregularity in the second cleavage than in Cryptobranchus.

Stage 3: (figs. 65 to 72 and 206). The third cleavage furrows appear about five hours after the beginning of the second; hence the interval is shorter than that between the first and second. At the time when the third furrows begin, the first furrow has usually reached or passed the equator.



Figs. 65 to 72 Types of third cleavage of Crytobranchus alleghenìensis. All the figures are of the upper hemisphere except figure 66 which represents the lower hemisphere of the egg shown in figure 65. In no case does any cleavage furrow except the first reach the lower pole. All the figures are camera drawings of preserved material. × 3½.

As the cleavage furrows invade the more heavily yolk-laden lower hemisphere they become comparatively faint except at their extreme lower ends where they broaden out. During this stage the second and third cleavage furrows are in general broader than the first.

At the stage represented in figure 65, when the third furrows are well established but extend only a short distance from their respective points of origin, the first cleavage furrow has reached the lower pole where its ends unite. The first cleavage furrow thus becomes superficially complete, thereby establishing the holoblastic character of the egg. That there is a strong meroblastic tendency is already apparent. In the region of the vegetal pole the first cleavage furrow is at first broad, but it later becomes narrow and faint.

With very few exceptions, the third cleavage furrows depart from the *second* furrow, at some little distance from its point of intersection with the first. In a previous paper (Smith '06) it was erroneously stated that the third cleavage furrows usually depart from the first furrow.

The third cleavage furrows ordinarily begin as two pits in the second furrow, equidistant from its point of intersection at the animal pole. From these two pits the third cleavage furrows ordinarily proceed in an approximately vertical direction (fig. 65), and do not become complete until a later stage (Stage 5).

From the time of the earliest appearance of the third cleavage furrows, the distances from the first cleavage furrow to their points of departure from the second remain unaltered; but the second cleavage furrow, originally straight, often becomes drawn into a zig-zag line, as shown in the figures.

As will be seen from a study of later stages, the third cleavage furrows rarely reach the vegetal pole, but as a rule extend obliquely in the lower hemisphere to join the *first* furrow at some distance from the lower pole (figs. 84 and 91 to 96). Hence the general statement may be made that the third cleavage furrows are intermediate between a true meridional and a true latitudinal cleavage but approach more nearly to the former type.

In the typical condition, the cleavage pattern has now lost its strictly radial, and acquired a biradial symmetry. I have purposely avoided the use of the word *bilateral* in this connection, not only because it does not fit the case so well as the word *biradial,* but in order to avoid the inference that the condition has anything to do with the bilateral symmetry of the future embryo. As will be seen by consulting the figures, this biradial condition of the cleavage pattern persists in the lower hemisphere throughout the late cleavage stages, and in some eggs enables one to identify the early cleavage furrows even after the beginning of gastrulation.

Deviations from the type in Stage 3 show a series of conditions connecting the typical one with a true latitudinal third cleavage. In such cases the third cleavage furrow proceeds more obliquely, and at an earlier stage joins the first nearer the animal pole (figs. 68 to 72). In some cases one or more of the third cleavage furrows are truly latitudinal (figs. 70 to 72).

Rare cases occur in which a third cleavage furrow originates at the animal pole, or from a first cleavage furrow (see fig. 67 for an example of the latter case); occasionally, a third cleavage furrow may reach the vegetal pole, or unite with a second cleavage furrow near the vegetal pole (figs. 94 and 95).

In comparing the third cleavage pattern of Cryptobranchus with that of other forms, one of the most obvious generalizations brought out is that a vertical third cleavage is characteristic of heavily yolk-laden and highly telolecithal eggs: e.g., the squid (Watase '91); Amia (Dean '96, Whitman and Eycleshymer '97); Lepidosteus (Dean '95, Eycleshymer '99); Acipenser (Dean '95); Ctenolabrus (Agassiz and Whitman '84); Serranus (H. V. Wilson '91); Ceratodus (Semon '00 and '01); Lepidosiren (Kerr '00 and '09); Cryptobranchus japonicus (deBussy '04 and '05); and the pigeon (Blount '07). But the rule is not absolute; concerning the third cleavage of Necturus, Eycleshymer ('04) says: "In most cases the cleavage grooves are irregularly formed and it might be said that the variations are so numerous and so diverse that a special description must be written for each egg." From this statement and an inspection of his figures (see also Eycleshymer and Wilson '10), it appears that a type cannot be recognized for the third cleavage of this egg; that the irregularity is greater than in the case of Cryptobranchus and that there is a more marked tendency for the third cleavage furrows to come in latitudinally. My material for the very early cleavage stages of Necturus is too scanty to enable me to form any conclusion based on direct observations, but some unpublished figures of the early cleavage of Necturus drawn from the living egg by

Prof. Bashford Dean give confirmatory evidence of the irregularity of the third cleavage furrows. In Desmognathus the third cleavage furrows were vertical and regular in the few eggs studied by Wilder ('04); the third furrows depart from an earlier cleavage furrow at some distance from the animal pole. But Hilton ('04 and '09), who examined a considerable number of eggs of Desmognathus in this stage, states that this regular and vertical form of cleavage occurred in only two or three eggs; in the others the third cleavage was irregular. In Diemyctylus (Jordan '93) there is still greater irregularity in the third cleavage than is recorded for Necturus or Desmognathus: "With the completion of the second furrow all consistent regularity is at an end."

In eggs less heavily yolk-laden, as in Amblystoma (Eycleshymer '95) and the frog, the third cleavage is latitudinal.

Especially interesting from a comparative point of view are Budgett's observations on the cleavage of the crossopterygian Polypterus as given by Kerr ('07). "From Budgett's pen and ink sketch . . . . we can see that the segmentation is at first characterized by its almost absolutely equal character. We may infer with considerable certainty that the two meridional furrows are succeeded by a latitudinal one which is practically equatorial." The egg of Polypterus is small, having a diameter of a little over one millimeter.

In urodeles we find a condition intermediate between the vertical third cleavage characteristic of the fishes generally, and the latitudinal third cleavage of the anura. In Cryptobranchus the vertical type prevails; in Desmognathus, Necturus and Diemyctylus there is increasing irregularity; in Amblystoma the third cleavage is latitudinal. The possible phylogenetic significance of the cleavage of Crytpobranchus will be considered later.

As a rather general rule, in eggs in which the third cleavage is usually vertical, the third furrows depart from the second rather than from the first or from the animal pole. As has already been seen in the case of Cryptobranchus allegheniensis, this rule is by no means absolute; but in general it applies also to the squid, and to the teleosts (e.g., Ctenolabrus, Serranus). DeBussy ('04 and '05) has described the cleavage stages of

Cryptobranchus japonicus; his material was meager and lacked first and second cleavage stages. He states ('05) that in the five eggs examined in the third cleavage stage, all the third furrows are approximately meridional. His figures ('04) represent the third furrows departing most frequently from the first cleavage furrow, sometimes from the second, sometimes from the animal pole. In the urodele Hynobius (Kunitomo '10) the cleavage pattern in this stage resembles that of Cryptobranchus allegheniensis, except that the third furrows do not so often depart from the second furrow. In Amia, Whitman and Eycleshymer ('97) describe the third cleavage furrow as follows:

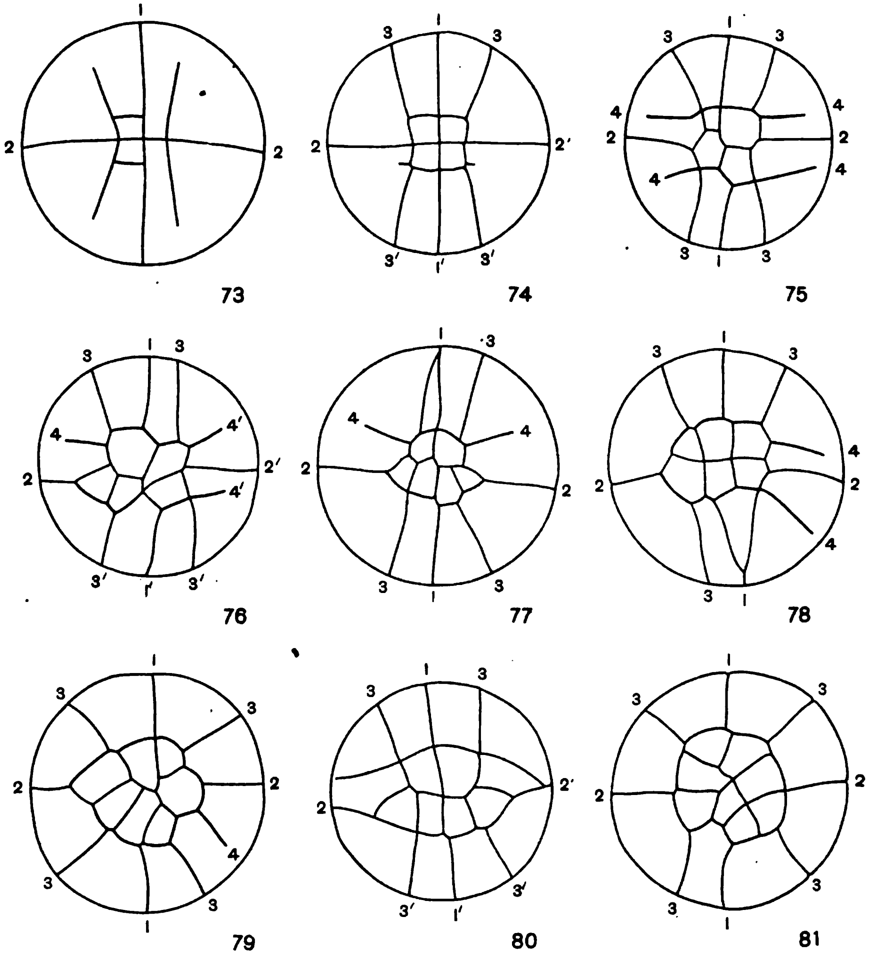
> In the majority of cases they are vertical . . . . They generally all depart from one or the other of the first two meridionals, thus giving rise to a distinct bilateral appearance . . . . It oftens occurs that one or more of the set depart from the first meridional, while the rest depart from the second, or *vice versa*.

Stage 4: (figs. 73 to 84 and 207 to 209). This stage is characterized by the appearance of the fourth cleavage furrows, giving, when complete, sixteen cells. As will appear from the following observations, the number of micromeres is not constant, but varies from four to eight.

The fourth cleavage furrows appear about four hours after the beginning of the third, and about thirty-nine hours after fertilization. Ordinarily they begin as two grooves, cutting the *first* cleavage furrow at right angles, on each side of the second and a short distance from it (figs. 73 and 74). Thus in position and direction the fourth cleavage furrows alternate with the third, which cut the *second* at approximately right angles. The fourth cleavage is the first one to cut off micromeres from macromeres, and the division is very unequal.

In a given lot of eggs the sixteen-cell stage is reached quite uniformly at the same time, but with so much variation in the direction of the fourth cleavage furrows that at first sight no uniformity is recognizable. By the study of a large number of eggs the following generalizations are established:

(a) In each quadrant, one micromere is cut off between the first and second cleavage furrows, giving in each egg a minimum of four micromeres (fig. 74).



Figs. 73 to 81 Fourth cleavage of Cryptobranchus alleghenicnsis, × 4⅔. All the figures are of the upper hemisphere. Figure 73 and 74 are drawn from the living egg; the others are camera drawings from preserved material. Figures 73 and 74 represent early stages of fourth cleavage; figure 80 is from the same egg shown in figure 74, representing the condition three hours later. Figure 81 is drawn from the egg photographed for figure 209.

(b) From the third cleavage furrows the remaining four parts of the fourth cleavage furrows may continue latitudinally, forming a complete circle or oval enclosing eight micromeres (fig. 81); or one or more of these four parts may continue approximately parallel to the second cleavage furrow, extending vertically and increasing the number of macromeres instead of cutting off micromeres (figs. 75 to 80). Thus while the total number of cells is always sixteen, the number of micromeres varies from four to eight.

We thus get as one extreme type an approximately latitudinal fourth cleavage furrow; as the other extreme a fourth cleavage furrow divided into two separate grooves, one on each side of the second furrow and approximately parallel to it and to each other. Between these two extremes we find examples of all possible intermediate conditions.

With regard to the manner of fourth cleavage, eggs of this stage may be classified into five types, depending on the number of micromeres present. For the purpose of such a classification, irregularities in the third cleavage must be allowed for: in cases where the third cleavage has come in diagonally or latitudinally to cut off a small cell, such a cell is divided by the fourth cleavage into two small cells, of which only the one nearer the animal pole is to be counted as a micromere (fig. 77).

To determine the mode, twenty-five eggs were examined in the sixteen-cell stage, and the results tabulated as follows:

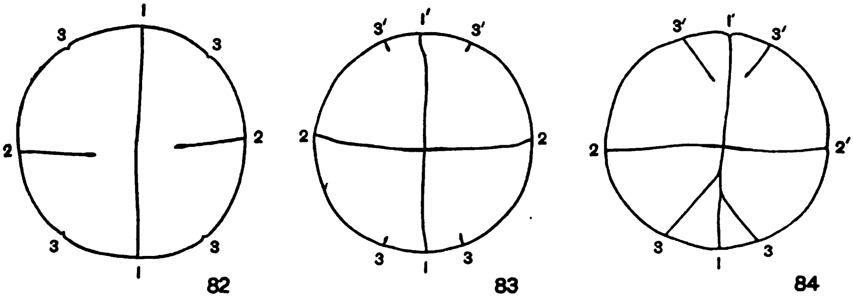
Number of micromeres	8	7	6	5	4
Number of cases	4	7	8	3	3

The table shows that the most frequent manner of cleavage is intermediate between the two extremes described.

In the majority of cases the micromeres are arranged with considerable regularity in two parallel rows, separated by the second cleavage furrow (see especially figs. 78 and 80). This is the necessary result of the biradial symmetry instituted by the normal mode of third cleavage, providing there is no extensive shifting of the micromeres. The condition reminds one of the cleavage pattern of the corresponding stage of the teleost egg.

Through a shifting of the micromeres, the biradial symmetry of the cleavage pattern of the blastodisc is usually interfered with (figs. 75 to 81). In this region, the first and second cleavage furrows become irregular and broken to an extent never observed in earlier stages. Outside of the region of the micromeres, the biradial pattern of cleavage is retained.

In this as in other cleavage stages the most recent furrows, and especially the most recent portions of such furrows, are in general quite noticeably the widest. This fact once established may be made use of in connection with other evidence to identify cleavage furrows. The broadening of the ends of the vertical furrows as they invade the lower hemisphere is a fairly constant feature of the cleavage; as shown by unpublished drawings from living material by Prof. Bashford Dean, it is also well expressed in the eggs of Necturus.



Figs 82 to 84 Lower hemispheres of fourth cleavage stages of Cryptobranchus allegheniensis. All the figures are camera drawings of preserved material. × 4⅓..

Fig. 82 Lower hemisphere of the egg shown in figure 208.

Fig. 83 Lower hemisphere of the egg shown in figure 76. This figure would serve equally well to represent the lower hemisphere of the egg drawn for figure 75.

Fig. 84 Lower hemisphere of the egg represented in figure 80.

In the majority of eggs of this stage, the second cleavage furrows have reached the lower pole, and the third furrows have just passed the equator (figs. 82 to 84). The second cleavage furrows intersect the first at right angles at the lower pole. For some distance on each side of the pole the second cleavage furrows are for a time markedly wider than the first. The second furrows are further distinguished by the fact that the third furrows run closer to them than to the first. In the latter part of this stage the third furrows sometimes become complete (fig. 84), as a rule joining the first at some distance from the pole.

The biradial pattern of cleavage is thus preserved in the lower hemisphere, and throughout the later cleavage stages affords a trustworthy means of distinguishing first and second cleavage furrows in this region.

The fate of the fourth cleavage furrows that proceed vertically must be studied in later stages. They usually join the second furrow before reaching the lower pole (figs. 92 and 96).

In a given egg the micromeres vary somewhat in size; but a comparison of seventeen carefully drawn camera figures, and the examination of a large number of additional eggs, lead to the conclusion that in this stage there is no regularity in the distribution of large and small cells among the micromeres.

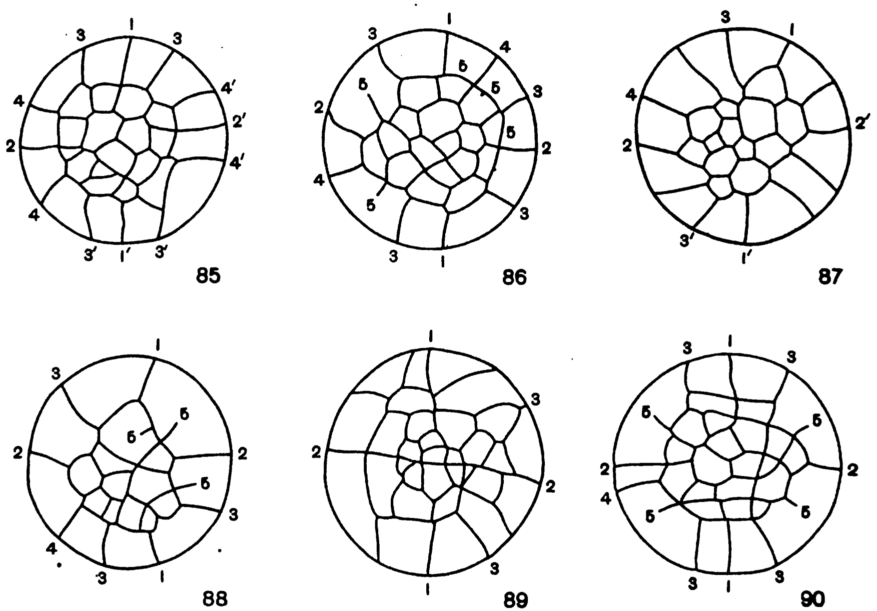
DeBussy's ('04) single figure of the fourth cleavage stage of Cryptobranchus japonicus shows six micromeres surrounded by an approximately circular cleavage furrow, and two recent furrows extending for a short distance vertically.

In Desmognathus, according to Wilder ('04), the fourth cleavage is latitudinal; this conclusion was based on the study of material very limited in amount. Hilton ('09) states that in a large number of eggs he has found only a few which exhibit so regular a type of cleavage as described in Wilder's eight cell and later stages.

In Hynobius (Kunitomo '10), the fourth cleavage furrows are more uniformly latitudinal than in Cryptobranchus alleghenienis. In Necturus (Eycleshymer '04; Eycleshymer and Wilson '10) and Diemyctylus (Jordan '93) a type is no longer recognizable.

In Ceratodus (Semon '00 and '01) the fourth cleavage is latitudinal. Amia (Dean '96; Whitman and Eycleshymer '97) and Lepidosteus (Dean '95; Eycleshymer '99) resemble the type with four micromeres described for Cryptobranchus alleghenienis.

Stage 5: (figs. 85 to 96; 210 and 211. This stage is reached about four hours later than Stage 4. It is characterized by the presence of the fifth cleavage furrows, giving a maximum of thirty-two cells, some incompletely divided. More than half of these cells are micromeres.



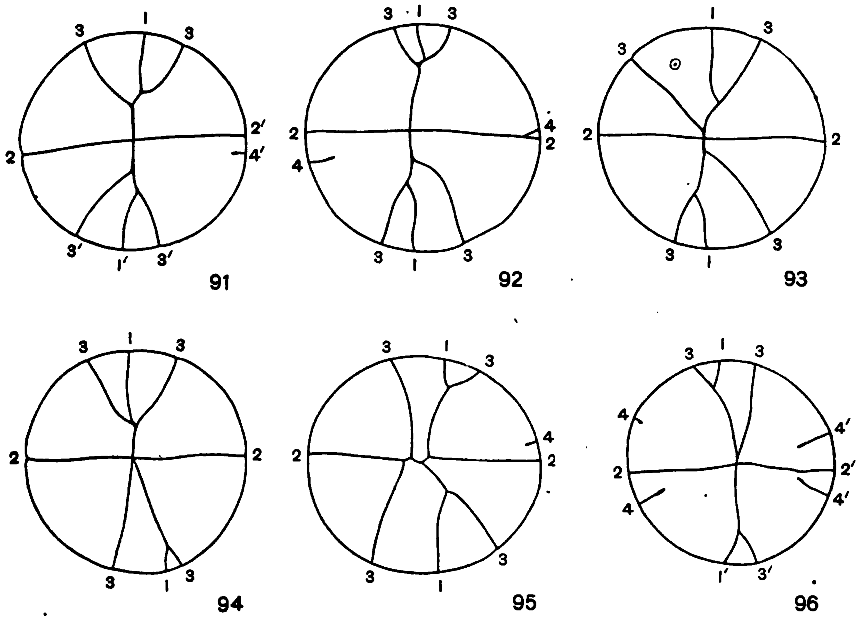
Figs. 85 to 90 Upper hemispheres of eggs of Cryptobranchus alleghenicnsis in the fifth cleavage stage. All the figures are camera drawings from preserved material. Figure 86 is drawn from the egg photographed for figure 211, and figure 87 from the egg photographed for figure 210. × 4⅔.

The careful study of a large number of eggs emphasizes irregularity in this cleavage and the absence of a well established type. Two sets of fifth cleavage furrows are often recognizable: an inner, within the former region of micromeres, and an outer, just outside of this region. Either set may be, in whole or in part, vertical, latitudinal or oblique (see especially figure 85 for an example of outer, and figure 89 for an example of inner latitudinal cleavage furrows). A study of the most regular cases of cleavage described under Stage 6 shows that in these eggs the fifth cleavage furrows must have come in with greater regularity than in any eggs directly observed in the fifth cleavage stage: these fifth cleavage furrows are almost uniformly vertical, thus preserving the regular alternation in the direction of the furrows.

By the shifting of micromeres the biradial symmetry due to the manner of third cleavage is usually lost in the blastodisc, and unless the egg has been kept under continuous observation it becomes in most cases impossible to trace the first and second cleavage furrows entirely through the region of micromeres.

In preserved material, nuclei are visible from the surface in some of the micromeres of this and the following stages, indicating that these cells are becoming flattened out.

As already noted, in this stage if not in the preceding one, the third cleavage furrows become complete, usually joining the first at some distance from the pole (figs. 91 to 96). This apparent avoidance of the pole by the third cleavage furrow is doubt-



Figs. 91 to 96 Lower hemispheres of eggs of Cryptobranchus allegheniensis in the fifth cleavage stage. All the figures are camera drawings from preserved material. Figure 93 shows a persistent sperm pit (see Part I, Smith '12). × 4⅔.

Fig. 91 Lower hemisphere of the egg whose upper hemisphere is shown in figure 87.

Fig. 96 Lower hemisphere of the egg whose upper hemisphere is shown in figure 85. The fourth cleavage furrows have extended further than is usual in eggs of this stage.

less the mechanical result of the location of the earlier course of the third furrows nearer to the first than to the second; they swerve from the vertical toward the nearest existing cleavage furrow. An analogous pattern may sometimes be observed in the cracking of the corners of a section of cement walk.

I have observed this biradial cleavage pattern in corresponding stages of the lower hemisphere of occasional eggs of Necturus; it is clearly expressed in the cleavage of Desmognathus as figured by Wilder ('04) and Hilton ('04 and '09).

The same tendency to join the nearest existing vertical furrow is shown by those fourth cleavage furrows, as a rule not yet complete, that come in vertically. They usually join the second furrow, at a much greater distance from the lower pole than the intersection of the third with the first.

In the vicinity of the vegetal pole, both first and second cleavage furrows are now only faintly expressed.

In about half the eggs of this stage cell division has proceeded a little more rapidly on one side of the egg than on the other; the cells are smaller in surface view, more numerous, and the cleavage furrows are more uniformly complete (figs. 87, 88 and 210). Thus there is an excentric development of the blastodisc, whereby a condition of bilateral symmetry in the cleavage pattern is produced. This excentric development is a more constant feature in the stages immediately following. The question naturally arises whether this bilateral symmetry in the cleavage pattern has any morphogenetic significance: is it the outward expression of the establishment of the permanent bilateral symmetry and antero-posterior differentiation of the embryo? In other words, does the axis of bilateral symmetry in the cleavage pattern fall in the median plane of the future embryo? This question will be considered in a later paper, in connection with the study of the internal development.

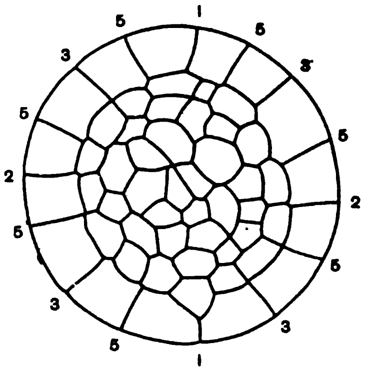
Such an excentric development of the blastodisc has been described for Amblystoma and Necturus by Eycleshymer ('95 and '04), who cites similar observations on other vertebrates by various writers. Eycleshymer speaks of a "second area of accelerated cell division" as distinguished from the primary area of cell division at the animal pole. In Cryptobranchus what happens seems to be a shifting of the most active area of cell division to an excentric position in the blastodisc; hence I have preferred to speak of it merely as a process of excentric development.

No constant relation exists between the axis of bilateral symmetry due to excentric development and the original direction of the first cleavage furrow as shown by those portions of it that have not undergone shifting. The two may coincide (fig. 88); they may be at right angles to one another; they may be oblique (fig. 87).

As already indicated, in this stage the cleavage pattern of Necturus bears a strong resemblance to that of Cryptobranchus allegheriensis, but there is this marked difference: the third cleavage furrows of Necturus, when vertical, usually join the first at a greater distance from the vegetal pole, in the region of the equator. In most eggs of Necturus examined in this stage only the first two cleavage furrows extend into the lower hemisphere; these usually meet at right angles at the vegetal pole. Thus the cleavage of Necturus in this stage seems to show an even stronger tendency toward the meroblastic condition. But this is merely a consequence of the tendency for the third cleavage furrows to come in obliquely or latitudinally; a comparison of later stages shows that the meroblastic tendency is in reality a trifle less strongly expressed in Necturus (figs. 107 and 108) than in Cryptobranchus.

In Amia (Dean '96; Whitman and Eycleshymer '97) the fifth cleavage furrows appear in two sets: an outer set cutting the eight macromeres latitudinally; and an inner set cutting the four micromeres in a horizontol plane, hence not visible from the surface.

Stage 6: (figs. 97 to 102 and 212 to 214). This stage is characterized by the presence of the sixth cleavage furrow, giving a maximum of sixty-four cells, some of the macromeres being incompletely divided. Considerably more than half the cells are micromeres; these occupy an area whose diameter extends over only about 90° of the circumference of the egg. Hence the mero-



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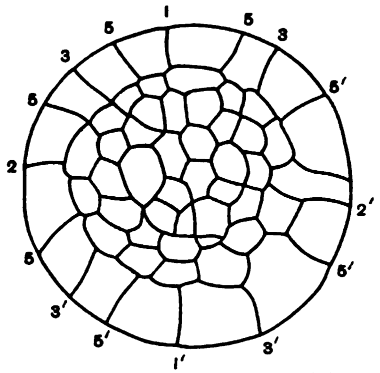

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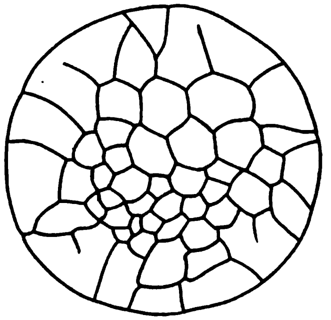

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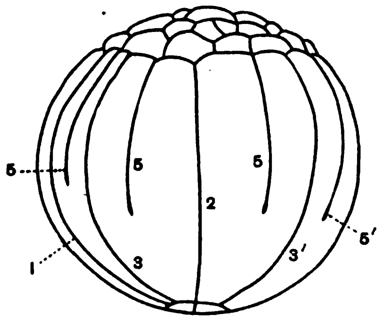

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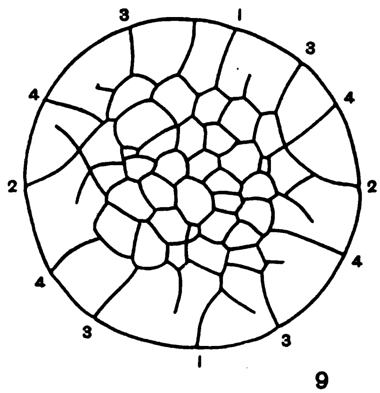

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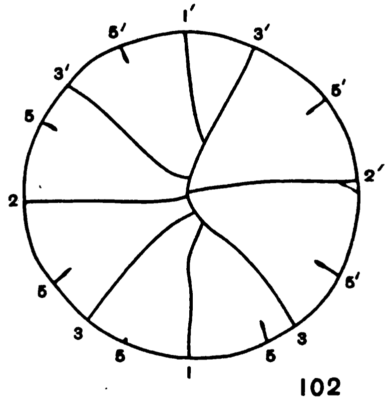

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Figs. 97 to 102 Sixth cleavage (Stage 6) of Cryptobranchus alleghenensis. Figures 100 to 102 show upper hemisphere, equatorial view, and lower hemisphere, respectively, of the same egg. All of the figures are camera drawings from preserved material. Figure 98 is drawn from the egg photographed for figure 212. × 7.

blastic tendency is strongly expressed. This stage is reached about four hours later than the beginning of the preceding stage.

A description of a few individual eggs will best indicate the characteristics of this cleavage.

Out of about fifty eggs studied, the one represented in figure 97 shows the greatest regularity of cleavage in the upper hemisphere. This condition must have been reached by a fairly constant alternation of vertical and latitudinal cleavage furrows. This alternation of cleavage furrows carried out with completeness and geometrical precision would give a total of sixty-four cells, consisting of forty-eight micromeres and sixteen macromeres; the micromeres would be arranged in three concentric rows, each containing sixteen cells. In the egg under consideration, this condition is realized in the outer row of cells, which is quite regular and contains the theoretical number, sixteen. But the total number of micromeres is only thirty-nine, hence a deficiency must exist in the central portion of the blastodisc, or some divisions in this region must have taken place horizontally. Sections show that no horizontal divisions have taken place in this region; on the other hand divisions parallel to the surface have sometimes occurred in the marginal row of micromeres. Therefore cell division is taking place more rapidly in the marginal than in the central region of micromeres—a condition which may be the beginning of that accelerated development of the margin, the later expression of which is almost wholly internal.

A study of other eggs showing a fairly regular alternation of cleavage furrows gives additional evidence for this interpretation (e.g., fig. 100, representing an egg with 39 or 40 micromeres). While eggs with this degree of regularity in the cleavage pattern are the exception rather than the rule, it is felt that evidence derived from them is especially trustworthy; for in such eggs the equilibrium in the rhythmic alternation of the direction of cell division has been best maintained, and the rather uniform lagging-behind of the divisions of the cells in the central area would seem to be the expression of a normal tendency in the life of the embryo. In eggs which show disturbances in this equilibrium, discordant factors are more likely to be present to obscure the normal expression of the course of development.

The sixth cleavage furrows of the outer set, when latitudinal, divide the macromeres very unequally, cutting off additional micromeres. The number of micromeres, and the extent of the blastodisc, is increased by such latitudinal divisions; the number of macromeres is increased by the sixth cleavage furrows only when these come in vertically.

In a few eggs, as the one shown in figure 99, there is a marked tendency for the sixth cleavage furrows to come in vertically. Here, as noted in an earlier stage, the embryo seems to be oscillating between two possible modes of cleavage; but the tendency to preserve the regular alternation of cleavage furrows is usually the stronger.

The most marked tendency to vary from the regular pattern of cleavage occurs along the line of excentric development of the blastodisc (figs. 98 and 212), as described under Stage 5. The majority of eggs exhibit this tendency in some degree.

We have then, in the cleavage pattern of this stage, two tendencies toward differentiation of the blastodisc: (a) an accelerated cell division in the marginal portion, pointing toward the formation of the germ ring; and (b) an accelerated cell division about a radius of the blastodisc, giving a condition of bilateral symmetry.

DeBussy's figure ('05, fig. 10) representing the blastodisc of an embryo of Cryptobranchus japonicus with forty micromeres strongly suggests excentric development; on one side of the first cleavage furrow only three cleavage furrows reach the equator, on the other side nine. But the author remarks (p. 530) that he has observed no secondary center of accelerated cell division such as has been described by Eycleshymer for Necturus.

A comparison with earlier stages shows that there is an increasing tendency for the micromeres, following a familiar law of developmental mechanics, to lose their original quadrangular or triangular outline and become hexagonal.

In the living egg, the roof of the segmentation cavity is somewhat translucent, and spaces communicating with the cavity beneath sometimes appear between the cells. Evidently the roof consists of a single layer of flattened cells; this inference is confirmed by the study of sections.

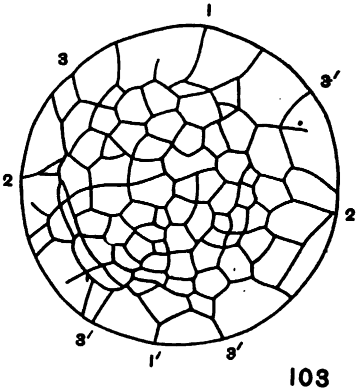
On account of the slow cleavage and relative stability of the macromeres, there is little change in the cleavage pattern of the lower hemisphere. An advance is shown in that the vertical fifth cleavage furrows have invaded the lower hemisphere (fig. 102). Those fourth cleavage furrows that proceed vertically are seldom complete in this stage, but sometimes are found joining an earlier vertical furrow at a considerable distance from the vegetal pole.

In this stage the most regular type of cleavage pattern of Cryptobranchus bears a striking resemblance to the corresponding stage of Amia (Dean '96; Whitman and Eycleshymer '97).

Since Stage 6 of the egg of Cryptobranchus best serves to illustrate the fundamental characteristics of the cleavage pattern, particularly with regard to the relative size of the micromeres and macromeres, at this point a comparison may well be made with the dipnoi and crossopterygii. The general anuran or urodele character of the cleavage of the dipnoan egg is apparent in all existing genera: Ceratodus (Semon '00 and '01); Protopterus (Budgett '01; Kerr '09); and Lepidosiren (Kerr '00, '01 and '09). With respect to inequality in the cleavage, Lepidosiren in particular closely approaches the condition in Cryptobranchus and Necturus. The cleavage of Polypterus (Kerr '07) bears a general resemblance to that of Amblystoma and the frog.

Stage 7: (figs. 103 to 105; 215 and 216). This stage is characterized by a doubling of the number of cells found in the preceding stage, and by a slight extension of the region occupied by the micromeres. The stage is reached about four hours later than Stage 6.

Figures 103 and 104 show a fairly representative egg in this stage. The cells in the region of the animal pole are markedly larger than the other micromeres. This condition may be due to one or both of two factors: (a) the flattening of the cells composing the roof of the segmentation cavity; (b) a slower rate of division in these cells, as noted in Stage 6. There is marked



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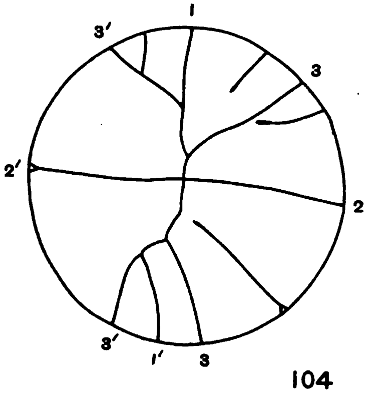

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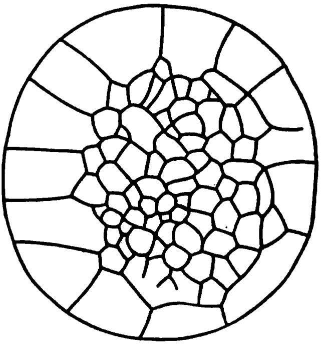

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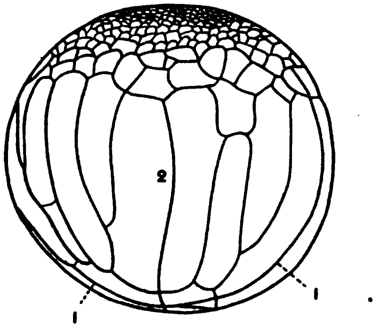

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Figs. 103 to 105 Surface views of eggs of Cryptobranchus allegheniensis in Stage 7, showing cleavage furrows. Figures 103 and 104 represent upper and lower hemispheres, respectively, of the same egg. Camera drawings from preserved material. × 7.

Fig. 106 Equatorial view of an egg in Stage 8, showing cleavage pattern. Camera drawing from preserved material. × 7.

activity in cell division in an area excentrically situated, though this is not so apparent in the particular egg under consideration as in some other eggs in the same stage. In the lower hemisphere, the biradial character of the cleavage pattern is preserved and accentuated.

In the egg represented in figure 105 we see the beginning of a process of fundamental importance in the further history of the embryo—the phenomenon of *immigration of cells from the single-layered roof of the segmentation cavity.* In a surface view, it is evident that some of the cells in the excentrically situated area of most rapid cell division are partially submerged. They are not merely smaller superficially than the other micromeres, but are sunken below the general surface and present the appearance of being crowded inward. Their condition will be further described in a consideration of the internal development; their later history and fate form an important phase of the process of embryo-formation.

In most eggs of this stage, at the margin of the blastodisc oblique furrows (probably fifth cleavage furrows) occasionally cut off cells intermediate in size between micromeres and macromeres. In the lower hemisphere some recent furrows, presumably fifth cleavage furrows, usually extend well toward the vicinity of the vegetal pole. The macromeres are, as a rule, long, narrow and wedge-shaped. On account of the segregation of most of the protoplasm within the region of the blastodisc, all latitudinal divisions of the macromeres are very unequal, cutting off new micromeres instead of increasing the number of macromeres. In the living egg, the roof of the segmentation cavity still appears somewhat translucent, and spaces sometimes occur between these cells; but neither of these conditions is so marked as in the preceding stage.

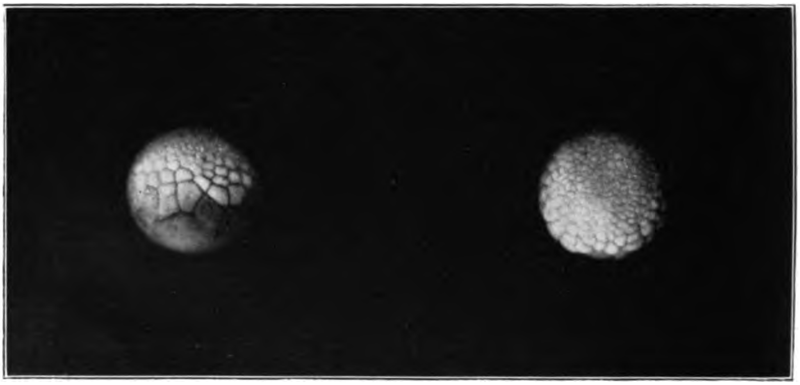
Stage 8: (figs. 106 and 217). This stage is reached about twelve hours later than Stage 7; it is best described by reference to the figures. The micromeres have become much more numerous and smaller; there is a slight extension of their area. There is a more gradual transition, or gradation in the size of the cells, between micromeres and macromeres. In the lower hemisphere the fifth cleavage furrows have, as a rule, become complete; they rarely reach the lower pole, but join an earlier furrow at some distance from the vegetal pole. Biradiality of the cleavage pattern still enables one, as a rule, to distinguish first and second cleavage furrows in this hemisphere.

In the upper hemisphere the excentric area of accelerated cell division noted in the preceding stages is usually quite marked.

In preserved material the nuclei of the micromeres are often easily distinguishable in surface views.

In the living egg, the roof of the segmentation cavity has become quite opaque, and the cells are compactly arranged. During the latter part of this stage a translucent condition begins to appear at the animal pole, indicating a thinning-out of the cells in this region, as in Stage 6; but this time the cells form a firm tissue, with no spaces between them.

In Necturus the cleavage furrows in the region of the vegetal pole are fainter than in Cryptobranchus; this condition is reversed in the upper hemisphere, where the micromeres are outlined far more boldly in Necturus (figs. 107 and 108) than in Cryptobranchus (both statements refer to preserved material, fixed by the same method).

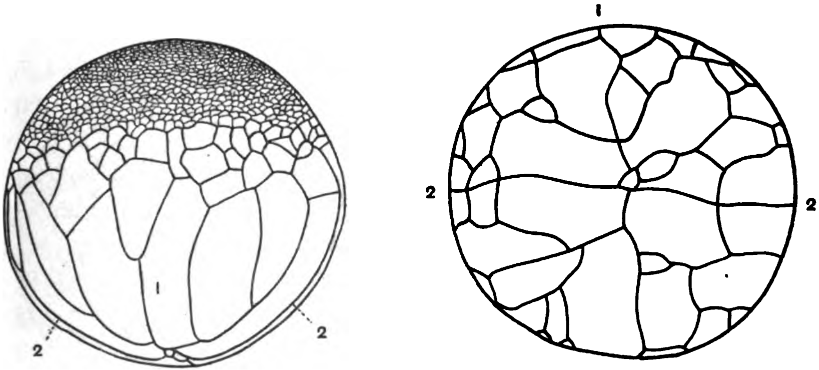


Figs. 107 and 108 Advanced cleavage stage of Necturus maculosus. Two views of a single egg, photographed after preservation. × 4.

Stage 9: (figs. 109, 110 and 218). This stage is reached about nineteen hours later than the preceding stage. Individual micromeres in the region of the animal pole are barely visible to the naked eye. The zone of transition between micromeres and macromeres has become broader and more marked. An excentrically situated area of accelerated cell division in the micromeres is only occasionally found in surface views of this stage.

In the living egg, the roof of the segmentation cavity appears as a translucent tissue throughout a circular area about 40 degrees in diameter in the region of the animal pole. This indicates a decided thinning-out of the cells of this region.

Biradiality of the cleavage pattern of the lower hemisphere still enables one to distinguish in many embryos, though not in all, the first and second cleavage furrows. Usually two or three cells quite small in surface view occur at the vegetal pole; they are quite characteristic of this and the following stage, but are sometimes found in the preceding stage. At the vegetal pole the cleavage furrows, both in living and preserved material, are sometimes both broad and deep, forming quite noticeable fissures; a similar condition is common in Amblystoma (Eycleshymer '95). In Cryptobranchus this condition is in marked contrast to the stage immediately preceding, in which the furrows in this region were faint. In Necturus, on account of the variability of the third cleavage furrows, the biradial pattern of the macromeres is not so clearly expressed as in the egg of Cryptobranchus.



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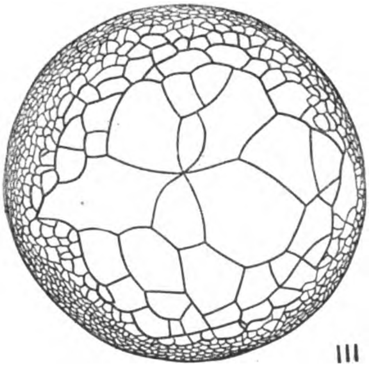
Figs. 109 and 110 Stage 9 of Cryptobranchus allegheniensis. Equatorial view and lower hemisphere of different eggs, showing cleavage pattern. Camera drawings from preserved material. × 7.

Stage 10: (figs. 111, 112 and 219). This stage, reached a day or two later than Stage 9, immediately precedes the beginning of gastrulation. The micromeres at the upper pole are invisible to the naked eye, and barely distinguishable with the magnification used for photographing (× 4). The area occupied by the micromeres extends approximately to the equator, though the broad zone of transition makes it difficult to define. In the vicinity of the vegetal pole, the cleavage furrows have again become faint; in many cases, in preserved material, they are distinguishable as fine lines lighter in color than the general surface, rather than as actual grooves. For the accurate study of these furrows in this and the following stage, a binocular microscope is usually required. When their boundaries are distinct, on account of their large size the macromeres are readily seen with the naked eye.



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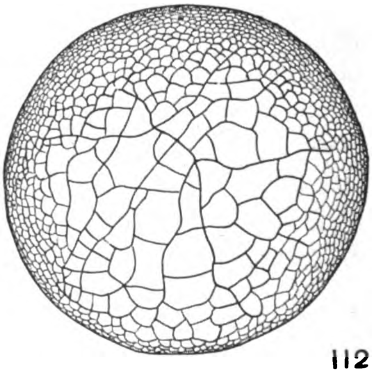

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Figs. 111 and 112 Lower hemispheres of two eggs of Cryptobranchus allegheniensis in Stage 10, showing cleavage pattern. The embryo shown in figure 112 is slightly older than the one represented in figure 111. Camera drawings from preserved material. In each egg, the lower pole as determined by gravity lies at the center of the figure; the vegetal pole, at the intersection of the first two cleavage furrows, is slightly above this point. The upper part of each figure represents the side on which the blastopore is to appear. × 7.

Usually, the cleavage pattern of the lower hemisphere retains enough of its earlier bilateral symmetry to enable one to distinguish first and second cleavage furrows. The vegetal pole, since it occurs at the intersection of the first two cleavage furrows, may in most cases still be determined quite accurately and conveniently by means of the cleavage pattern. As shown in figures 111 and 112, the vegetal pole is excentrically located in the area occupied by the macromeres; a more rapid multiplication of cells has occurred on one side of this area, so that on this side the micromeres and transitional cells approach nearer the vegetal pole. A meridian drawn through the vegetal pole and the center of the area occupied by the macromeres defines the axis of excentricity; this axis bears no constant relation to the first cleavage furrow and the axes of biradial symmetry determined by the early cleavage furrows. The biradial symmetry of the cleavage pattern is of course somewhat disguised by the more rapid multiplication of cells at one end of the axis of excentricity.

In this stage occurs a slight tilting of the morphological axis of the egg within a meridional plane determined by the axis of excentricity, so that the vegetal pole no longer coincides with the lower pole as determined by gravity. The vegetal pole is slightly uptilted on the side where the more rapid multiplication of cells occurs, hence the meridian defining the axis of excentricity passes also through the new pole determined by gravity. This new pole at first lies intermediate between the vegetal pole and the center of the area occupied by the macromeres; in later stages, through continued tilting of the egg in the same direction, it comes to lie beyond this center. Throughout the ensuing stages we must distinguish between the morphological axis of the egg as determined by the animal and vegetal poles, and the vertical axis determined by gravity. The method of locating the vertical axis, and exact measurement of the amount of rotation, will be given in the following stage.

If the egg be sectioned along the axis of excentricity the internal structure, to be described later, shows that this axis lies in the sagittal plane of the embryo; the side on which the small cells approach nearer to the vegetal pole is the one on which the blastopore is to appear. Thus the excentric position of the vegetal pole within the area occupied by the macromeres enables one to orient the egg with reference to future body regions.

In perhaps the majority of cases, the transition from large to small cells is more evenly graded on the side where it occurs nearest to the vegetal pole, than on the opposite side where it is characterized by a rather abrupt line of demarcation (figs. 111 and 112). This feature, when present, gives a true bilateral symmetry to the cleavage pattern of the lower hemisphere; the axis of this bilateral symmetry coincides with the axis of excentricity previously defined, hence lies in the sagittal plane of the embryo. The excentric position of the vegetal pole within the area occupied by the macromeres, and the bilateral character of the cleavage in this region, are more marked in many eggs taken inmediately after the beginning of gastrulation; these features are usually better expressed than in the eggs shown in figures 111 and 112, which were chosen because the distinctness of the early cleavage furrows enabled them to be drawn with the camera lucida. Schultze ('00, Taf. 11, fig. 12) has described a similar bilaterality in the late cleavage of the lower hemisphere of the frog's egg.

The question of the possible relation of the excentric and bilateral development of the lower hemisphere just described, to the excentric development of the blastodisc noted in previous stages, will be discussed in a later paper.

In the living egg, the roof of the segmentation cavity, though apparently thin, is not quite so translucent as in the preceding stage. It is, however, noticeably more translucent on the side toward the future blastopore, and on this side the transition to the opaque yolk cells is more abrupt.

During the late stages of cleavage, a tendency toward fading out of the cleavage furrows in the vicinity of the vegetal pole has been noted. In some individual cases this process has gone so far that the earlier cleavage furrows are lost to view, even when searched for with the binocular microscope. This tendency may be interpreted as due to a difficulty in sustaining the holoblastic method of cleavage in an egg so heavily laden with yolk. In the corresponding stages of Necturus, this tendency is even more marked. My study of the cleavage pattern of the lower hemisphere of the late segmentation stages of both Cryptobranchus and Necturus has been confined to preserved material, but Professor Dean informs me that he has noticed this merging of lower blastomeres in the late segmentation stages of the living eggs of Necturus. My Necturus material is not so favorable as the egg of Cryptobranchus for the study of the cleavage pattern in this stage, so a detailed comparison will not be attempted.

In the very late cleavage stages of Cryptobranchus japonicus, Ishikawa ('08 and '09) describes a shallow furrow ('Scheidewandfurche' or 'septal furrow') bounding the posterior margin of the roof of the segmentation cavity, parallel to the future blastopore. Such a furrow does not normally occur in this stage of Cryptobranchus allegheniensis, but a similar furrow makes its appearance shortly after the beginning of gastrulation.

B. Summary

The cleavage is holoblastic, but with great inequality in the size of the micromeres as compared with the macromeres.

Biradial symmetry of the cleavage pattern begins with the third cleavage stage. In the upper hemisphere, as a consequence of the shifting of the micromeres, this biradial symmetry is lost at about the fifth cleavage stage. In the lower hemisphere, because of the slow cleavage and stability of the macromeres, it persists until after the beginning of gastrulation, and in some eggs enables one to distinguish first and second cleavage furrows even after the blastopore has appeared.

An excentrically situated area of unusually small micromeres is apparent in surface views of most eggs in Stages 5 to 8 inclusive; the cleavage pattern of such eggs thus possesses bilateral symmetry.

In the sixth cleavage stage there is more rapid cell division in the marginal region of the micromeres than in the region of the animal pole. The later expression of this tendency is almost wholly internal.

In Stages 7 and 8 some of the superficially smaller micromeres are becoming submerged through a process of immigration.

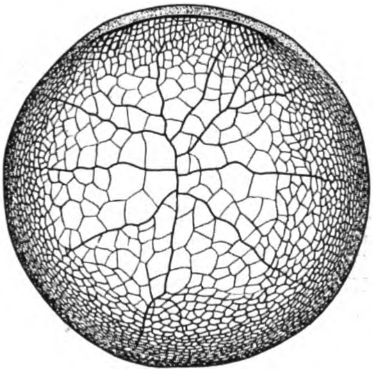
In the later cleavage stages there is a tendency for the cleavage furrows to become less distinct than formerly in the region of the vegetal pole, indicating a difficulty in sustaining the holoblastic character of the cleavage in an egg so heavily laden with yolk; the same tendency is observed in Necturus.

In late segmentation immediately preceding gastrulation the cleavage pattern enables one to predict the side on which the blastopore is to appear; the egg undergoes a slight rotation on a horizontal axis at right angles to the median plane.

VIII. GASTRULATION AND EARLY FORMATION OF THE EMBRYO

A. Description by stages

Stage 11: (figs. 113 to 137 and 220 to 222). This stage extends from the time of the first appearance of the blastopore as a short horizontal groove until its ends meet to form a complete circle. In eggs kept in their natural environment, gastrulation begins about seven days after fertilization and two days after the beginning of Stage 10.



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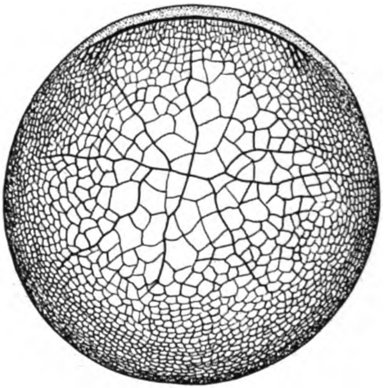

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Figs. 113 and 114 Lower hemispheres of two eggs of Cryptobranchus allegheniensis in an early gastrula stage, showing cleavage furrows. The vertical axis as determined by gravity lies at the center of each figure; the vegetal pole, at the intersection of the first two cleavage furrows, is about 7 degrees above the vertical pole. In figure 113 the first cleavage furrow lies approximately in the median plane of the gastrula; in figure 114 it is at right angles to this plane. Camera drawings, finished under the binocular, from preserved material. × 8.

The blastopore is first distinguished as a shallow irregular and broken horizontal groove two or three millimeters in length, lying about 15 degrees below the equator. It occurs at the upper limit of transitional cells between micromeres and macromeres, and its immediate site is distinguished by a rather abrupt demarcation between micromeres and distinctly larger transitional cells. The groove is started, not by a lining-up of cells and the union of cleavage furrows, as described by Eycleshymer ('95) for Amblystoma, but by the sinking-in of groups of entire cells at intervals along a narrow zone several cells in width; hence from its very beginning the process is not a splitting-apart of cells, but invagination. The groove soon becomes continuous and deepens by the inturning of cells along both margins.

After the groove has reached a length of three millimeters or more, the process of invagination becomes accompanied by one of overgrowth or epiboly: the dorsal lip grows slowly down over the cells transitional between micromeres and macromeres (figs. 113 to 115). As shown in figure 115, the transitional cells just within the horns of the blastopore are elongated as if compressed; here the cells line up and lengthen out at right angles to a line connecting the horns of the blastopore.

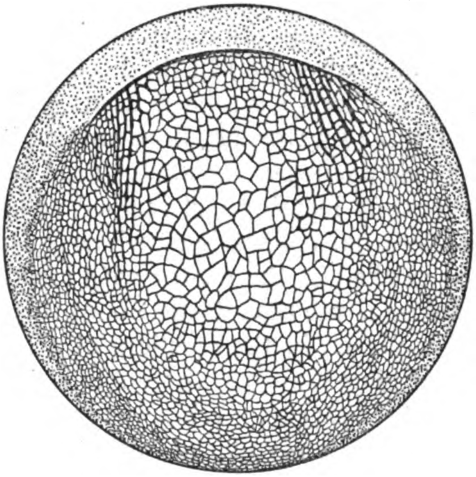


Fig. 115 Lower hemisphere of a gastrula of Cryptobranchus alleghenienisis, in a slightly later stage than the preceding, showing the lining-up of the cells within the horns of the blastopore. Freehand drawing from a photograph of preserved material. × 10.

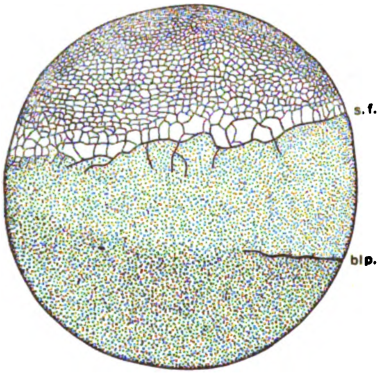
After the blastoporic groove has attained the form of a semicircle (fig. 133), a zone of rather abrupt demarcation between micromeres and transitional cells completes the circle begun by the crescentic blastopore; this zone marks the site of the ventral lip of the blastopore. A little later, the blastoporic groove extends rapidly along this line of demarcation, becoming an almost perfect circle, and enclosing a broad horseshoe-shaped band of transitional cells, within which lie the macromeres (fig. 138).

It has already been noted that a slight rotation of the egg on a horizontal axis has taken place in Stage 10, so that it is now necessary to distinguish between the morphological axis of the egg and the vertical axis determined by gravity, since the two no longer coincide. In the study of gastrulation this rotation must be taken into account, and some means must be found for measuring it. In studying the morphological features of the egg (position of blastopore, etc.) in their relation to the vertical axis, two general methods have been used: (a) the living egg, placed in a small vial of water, has been studied in side view and measurements made against a protractor used as a background; and (b) for accurately locating the vertical axis I have devised the following apparatus: a glass disc such as is used for an ocular micrometer was marked in the center with a small dot; a circle with a radius of 4 mm. was then drawn about this dot as a center. When this disc is placed in the eyepiece of a low-power microscope used in studying the eggs, the circle is just large enough to enclose the image of an egg. When the egg, immersed in water in a watch glass, is accurately placed so that its image is enclosed by the circle, the dot lies over the upper pole of the vertical axis; this point is then marked by puncturing with a hot needle. The operation was first tried on living eggs, which were then fixed for further study; but since with the living egg even a small puncture in this region usually causes the embryo to collapse during the subsequent process of fixation, in general this method is less satisfactory with living than with preserved material. On account of the usually perfect preservation of the form of the egg by the fixing fluid employed, the results obtained by marking preserved material seem fairly trustworthy, especially when spherical eggs are selected and a large number used. The position of the upper vertical pole, thus marked, gives a reference point for correlating the morphological features of the egg with the vertical axis; the measurements were made by means of camera drawings.

In making these measurements, it is especially necessary to guard against using eggs with an unusually large yolk plug, since this is one of the commonest abnormalities. Moreover, even in perfectly normal eggs there is considerable variation in the position of the blastopore, so that a large number of eggs must be studied and averages taken. The results obtained by the two methods agree closely.

Since the blastopore, at the time of its first appearance is only about 15 degrees below the horizontal equator and approximately parallel to it, the blastopore at first forms an arc of an imaginary circle whose diameter, measured along a meridian of the egg, is about 150 degrees. At the time when the blastopore has reached the form of a semicircle, this diameter measures about 125 degrees; when the blastopore has become a complete circle the average diameter, in normal embryos, is only 94 degrees. Therefore the crescentic blastopore forms an arc of a circle of steadily diminishing diameter; the lips of the blastopore, and the entire germ ring (to be described in a later paper), contract as they progress slowly downward over the egg.

In preserved material the cleavage pattern of the macromeres is still fairly well defined; by means of careful study with a binocular microscope it is usually possible to distinguish first and second cleavage furrows (figs. 113 and 114). This enables a direct comparison to be made between the direction of the first cleavage furrow and the median plane of the gastrula; this point will be discussed in a later paper. The identification of early cleavage furrows in this region is furthermore of importance in enabling one to determine the position of the vegetal pole, since this is located at the intersection of the first two cleavage furrows. Measurements show that at the time when the blastopore is first clearly established, the vegetal pole lies, on the average, 68 degrees below it, and 7 degrees above the lower pole of the vertical axis. At the time when the blastopore becomes a semicircle, the vegetal pole lies only 32 degrees below its dorsal lip; when the blastopore first becomes a complete circle the vegetal pole lies only 26 degrees below the dorsal margin of the yolk plug. During this time continued rotation of the egg has brought its



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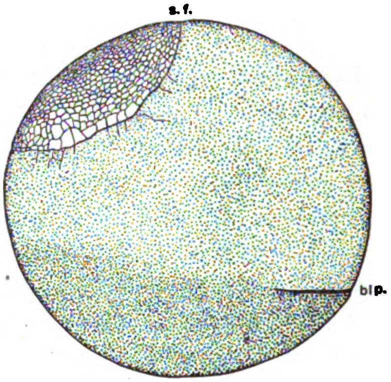

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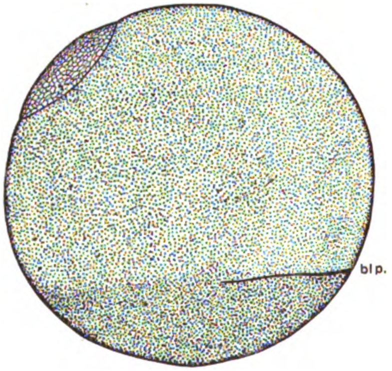

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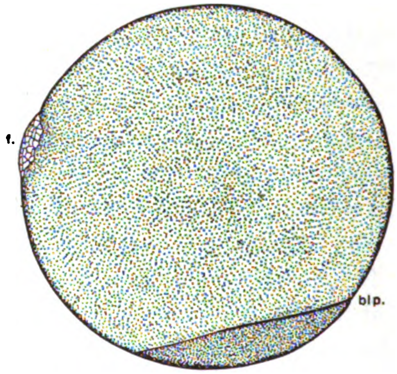

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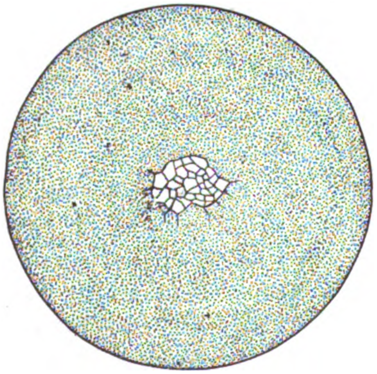

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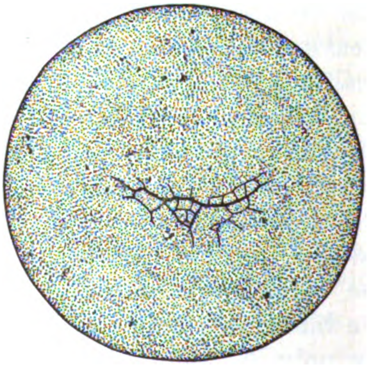

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morphological axis to an angle of 44 degrees from the vertical; the ventral lip of the blastopore now lies about 24 degrees beyond the lower pole of the vertical axis. These changes are set forth diagrammatically in figures 134 to 137.

Two quantitative results of considerable importance are brought to light through the study of these data: (a) the dorsal lip of the blastopore has grown downward over the yolk cells for a distance of about 42 degrees; (b) the egg has rotated in the opposite direction about 37 degrees from its position at the beginning of gastrulation, making a total rotation of 44 degrees. At first, overgrowth is more rapid than rotation; at the time when the blastopore has reached the form of a semicircle its dorsal lip is 43 degrees below the horizontal equator. Later, rotation is more rapid than overgrowth, and at the time when the blastopore has become a complete circle its dorsal lip has been carried back to a position 20 degrees below the horizontal equator, only 5 degrees lower than its original position in space.

The changes in the upper hemisphere visible from the surface during the establishment of the blastopore are remarkable, since they afford clues to many important processes within. In the living egg especially, because of the translucent character of the upper hemisphere, one is able to get total views of many phases of gastrulation, such as could not be obtained from serial sections except by means of reconstructions. Except where otherwise noted, the following description is based on the study of the living egg.

At the very beginning of the process of gastrulation, the nearly transparent roof of the segmentation cavity is of quite uniform

Figs. 116 to 121 Stage 11 (gastrula) of Cryptobranchus alleghenienis. Camera drawings from preserved material. In all the figures, the upper vertical pole as determined by gravity lies toward the top of the page; *blp.*, dorsal lip of the blastopore; *f.*, fenestra (roof of the blastocoele differentiated into a window-like structure); *s.f.*, septal furrow. × 7½.

Fig. 116 Lateral view of an early gastrula stage. The sharp differentiation of the fenestra is rather precocious in this egg.

Figs. 117 to 119 Lateral views of a characteristic series of later embryos.

Figs. 120 and 121 Antero-ventral views showing stages in the disappearance of the fenestra. Figure 120 is from the egg drawn for figure 119.

extent about the animal pole as a center, covering an area about 140 degrees in diameter. As gastrulation advances this clear area becomes encroached upon at its posterior margin (figs. 122 and 123) by the extension of the opaque material. Meanwhile the boundary of the roof of the blastocoele becomes more sharply defined; before the upgrowth of the postero-dorsal opaque region has reached the animal pole the margin of the blastocoele roof is usually bounded by a sharply defined furrow, the 'septal furrow' of Ishikawa (see below)—a characteristic and almost unique feature of the gastrulation of Cryptobranchus. The precise stage at which this furrow appears varies considerably in different eggs; figure 116 shows a case of unusually early appearance, figures 117, 125 and 126 a stage in which it is usually well established. Moreover, the distinctness of this groove varies greatly, particularly in eggs of different spawnings; in some lots of eggs the groove is established early and is very sharply marked, while in occasional lots of eggs it is almost absent.

The septal furrow appears first at the posterior margin of the roof of the segmentation cavity, then extends gradually around to its anterior margin; in its appearance and manner of extension

Figs. 122 to 133 Stage 11 (gastrula) of Cryptobranchus alleghenienssis. Freehand drawings of the living eggs, viewed by both transmitted and reflected light; the proportions of the various parts are checked by comparison with camera drawings of preserved material. The drawings are oriented with respect to the vertical axis determined by gravity. The roof of the segmentation cavity is nearly transparent; the roof of the gastrocoele is quite translucent, or slightly opaque in the regions containing mesoderm; heavily yolk-laden regions are decidedly opaque; *bc.*, roof of blastocoele; *blp.*, dorsal lip of the blastopore; *f.*, fenestra (roof of the blastocoele differentiated into a roof-like structure); *gc.*, gastrocoele; *m.*, region containing mesoderm. × 5.

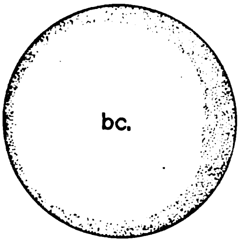
Figs, 122 to 124 Upper hemisphere, lateral view and lower hemisphere of an egg in the beginning gastrula stage.

Figs. 125 and 126 Upper hemisphere and lateral view of an egg a little later than the preceding.

Figs. 127 to 129 Postero-dorsal view, upper hemisphere and lateral view of an egg slightly later than the preceding.

Fig. 130 Upper hemisphere of a slightly later egg.

Figs. 130 to 133 Upper hemisphere, postero-dorsal view and lower hemisphere of an egg near the close of Stage 11 (shortly before the appearance of the neural groove).



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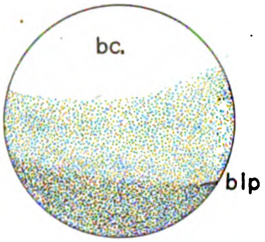

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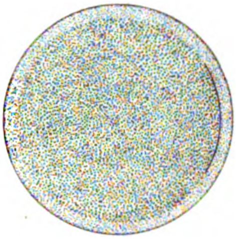

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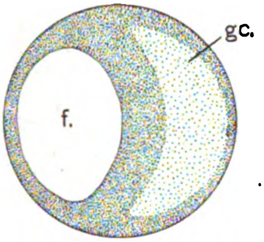

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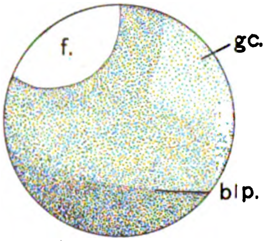

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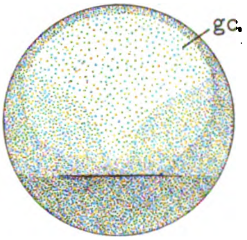

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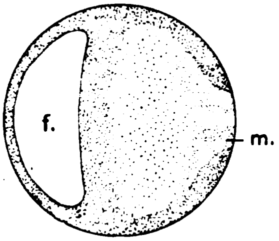

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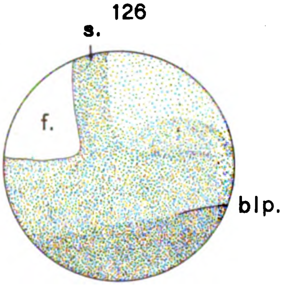

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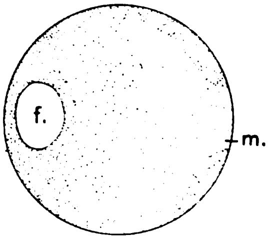

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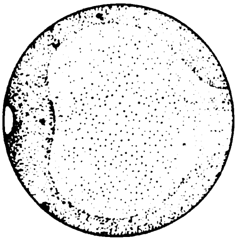

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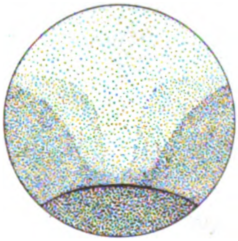

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it somewhat resembles a blastopore (fig. 221). Later, the groove becomes faint at its posterior margin, very pronounced at its antero-ventral margin (fig. 222). The area enclosed by the groove diminishes in size with its forward movement; it also becomes almost transparent. Since throughout the remainder of its history this area strikingly resembles a window, I shall refer to it as the *fenestra*.

In material fixed in a modification of the bichromate-acetic-formalin mixture (see Smith '12, Section III, Solution B) containing twice the usual amount of potassium bichromate, the fenestra is cut up into small polygonal areas separated by furrows that greatly resemble cleavage furrows (figs. 116 to 120 and 222; cf. Ishikawa, '08 and '09). These polygonal areas do not represent single cells; each comprises a group of several cells. The phenomenon is not entirely an artifact, since it often appears, though faintly, in the living egg. By this method of fixation the septal furrow is likewise accentuated.

Before describing the further history of the fenestra it is desirable to direct attention to some other changes in the upper hemisphere as observed in the living egg.

About the time that the fenestra becomes limited to the anterior half of the upper hemisphere by the upgrowth of the posterior margin of the opaque region, a translucent area, the roof of the gastrocoele, appears in this region of upgrowth (figs. 125 and 126). This translucent area is at first crescent-shaped; it is separated from the more transparent fenestra by an opaque band which is the outward expression of the septum separating the gastrocoele from the blastocoele.

As soon as the septum has advanced into the anterior half of the upper hemisphere, the translucency of the roof of the gastrocoele extends backward almost to the blastopore—evidently by the deepening of the gastrocoele in this region, admitting light. Meanwhile each postero-lateral margin of this region becomes bordered with a faint band of a slightly more opaque character—an effect due largely to the early mesoderm (figs. 127 to 129), though the entoderm is also concerned in producing it.

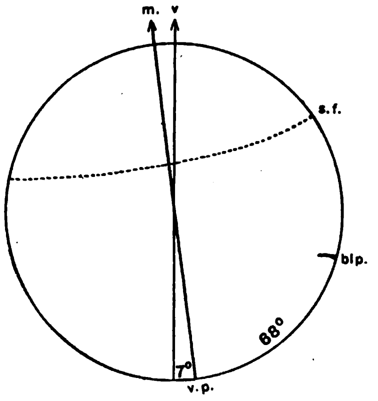
Later changes are concerned with the forward, or rather ventrad, progress of the septum and the increase in the extent of the translucent roof of the gastrocoele, with a correlated ventrad movement of the fenestra and a diminution of its area; there is a slight increase in the extent and opacity of the mesoderm (figs. 118 to 120 and 130 to 132). The fenestra finally closes just below the horizontal equator on the ventral side of the egg (fig. 121). The changes in the position and extent of the fenestra are shown diagrammatically in figures 134 to 137.

The foregoing detailed account of the progress of the septum as viewed from the exterior in the living egg of Cryptobranchus clears up whatever doubt may exist as to the significance of the 'shadowy area' described in the gastrula of Spelerpes by Goodale ('11) and confirms his suggestion as to the nature of this area.

Ishikawa ('08 and '09) describes in the early gastrula of Cryptobranchus japonicus a furrow bounding the roof of the blastocoele at its posterior margin, which he calls the 'Scheidewandfurche' or 'septal furrow.' As compared with the furrow of similar nature described above for C. alleghenіensis, it is earlier in making its appearance, since it antedates the blastopore. The area later enclosed by this furrow has been named by Ishikawa the 'Keimhohlensegment' or 'blastocoele-segment'; judging from his figures its later history is much the same as that of the corresponding structure, which I have preferred to call the 'fenestra,' in Cryptobranchus alleghenіensis.

The only mention of similar structures which I can find in the literature on other forms is a description by Hatta ('07) of a groove which he calls the 'boundary groove' in the gastrula of Petromyzon. As compared with the septal furrow of Cryptobranchus this groove is greatly exaggerated in Petromyzon, constricting the egg so that in some cases it assumes an hour-glass form.

As suggested by Hatta, the boundary groove or septal furrow is passive in origin, and a product of gastrulation. Similar conditions have produced it in two such widely separated forms as Cryptobranchus and Petromyzon; in each case the egg contains considerable yolk, and the roof of the blastocoele is unusually



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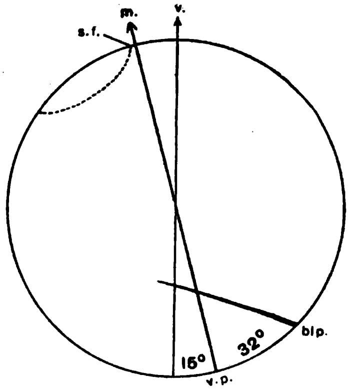

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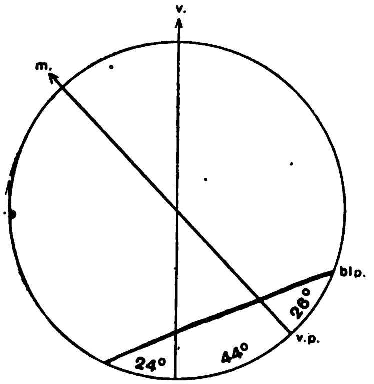

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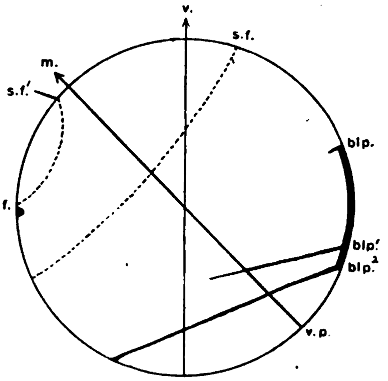

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Fig. 134 Diagram of an egg of Cryptobranchus alleghenïensis in the beginning gastrula stage viewed from the lateral aspect, showing average amount of rotation, and the positions of the beginning blastopore and the septal furrow; *blp.*, blastopore; *m*, morphological axis; *s.f.*, septal furrow; *v*, vertical axis determined by gravity; *v.p.*, vegetal pole.

Fig. 135 Similar diagram of a gastrula at the time when the blastopore reaches the form of a semicircle. Lettering as before.

Fig. 136 Similar diagram of a gastrula at the time when the blastopore first becomes a complete circle. Lettering as before.

Fig. 137 Combination of the preceding diagrams. The egg is shown in the position assumed at the close of the period considered. The black band indicates the amount of overgrowth of the dorsal lip of the blastopore (42 degrees); *blp.*, *blp'*, and *blp*^{2}, mark the successive positions of the dorsal lip of the blastopore; *s.f.* and *s.f.'*, successive positions of the septal furrow; *f*, position of the vanishing fenestra. Other lettering as in the preceding figures.

thin. As stated by Ishikawa, the polygonal figures formed on the surface of the blastocoele-segment (fenestra) are perhaps due to the pressure which produces the gradual diminution of its area; but the cells of the fenestra are not compacted together to any considerable extent, since the gastrocoele roof and wall merely grow under them.

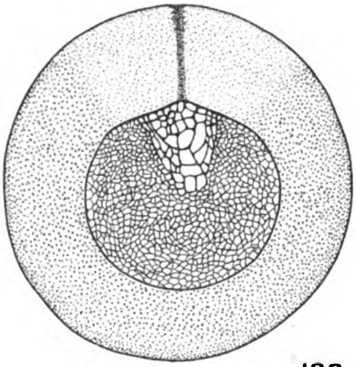
In view of the later history, it is evident even from surface views that in the stage shown in figures 116 and 123 the formative material for the embryo is mainly concentrated in the equatorial region as a broad band or zone of cells, wider in its posterior portion. As will be shown in the description of the internal structure, this equatorial zone as distinguished in surface views is only roughly comparable to the germ ring of fishes.

My material is lacking for the study of the early gastrula stages of Necturus; late gastrula stages differ from Cryptobranchus chiefly in that the blastopore earlier becomes a complete circle. In Spelerpes, according to Goodale ('11), no ventral lip is formed to the blastopore. As compared with urodele and anuran eggs in general, the blastopore of Cryptobranchus is late in closing; in its mode of gastrulation the egg of Cryptobranchus approaches more nearly the type observed in meroblastic eggs.

Stage 12: (figs. 138 to 150 and 223 to 225). This stage is characterized by the presence of the neural groove and is terminated by the appearance of the neural folds. The neural groove appears about three days after the beginning of gastrulation.

At the time of the earliest indications of the neural groove, the blastopore has just become a complete circle. At the close of the preceding stage it had a diameter of about 94 degrees; it now rapidly becomes smaller, so that before the appearance of the neural folds its diameter averages about 26 degrees (figs. 138 to 145).

During the early part of this stage the yolk plug is characterized by a broad crescent-shaped or horseshoe-shaped area of smaller cells lying ventrad and laterad to the macromeres (figs. 138 and 139). Along the lateral line of transition between the macromeres and these smaller cells, the cells appear compressed and exhibit a tendency to line up and merge their cleavage furrows (figs.



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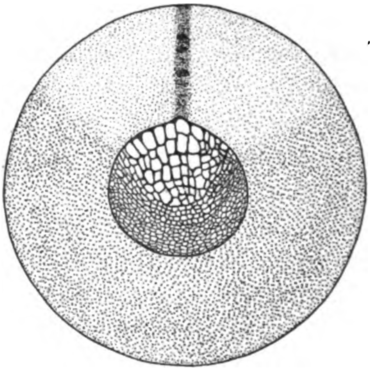

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Figs. 138 and 139 Posterior views of embryos of Cryptobranchus alleghenionsis in Stage 12, showing the condition of the blastopore and the cleavage furrows of the yolk plug. Camera drawings from preserved material. The embryos are not accurately oriented with respect to the vertical axis determined by gravity. × 7.

Fig. 138 Showing condition shortly after the appearance of the neural groove.

Fig. 139 A little later than the preceding.

138 to 142; cf. figs. 113 to 115). Toward the close of this stage, the smaller cells become completely overgrown by the ventral and lateral lips of the blastopore, leaving only the larger ones exposed (fig. 144); evidently overgrowth is now proceeding more rapidly at the ventral than at the dorsal lip of the blastopore. At the close of the stage the greatly reduced yolk plug lies entirely on the postero-dorsal side of the lower pole of the vertical axis.

During the earlier part of the stage under consideration the closing fenestra often persists as a pit or small tract of distinct

Figs. 140 to 145 Dorsal views of embryos of Cryptobranchus alleghenionsis in Stage 12, showing a series of stages in the development of the neural groove. Camera drawings from preserved material. The embryos are not oriented with respect to the vertical axis determined by gravity. × 7.

Fig. 140 Showing earliest appearance of the neural groove.

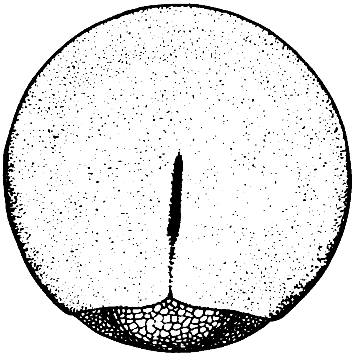
Fig. 141 Slightly later than the preceding.

Fig. 142 Slightly later than the preceding, showing segmented neural groove.

Fig. 143 Slightly later, segmented neural groove. See also figure 225 from the same embryo.

Fig. 144 Late neural groove.

Fig. 145 Showing the condition of the neural groove at the time of the first faint indications of the neural folds.



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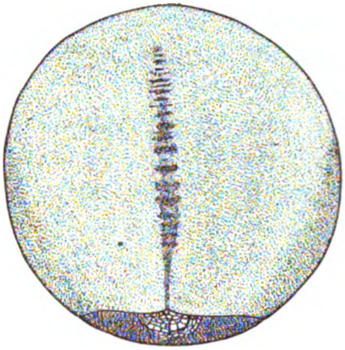

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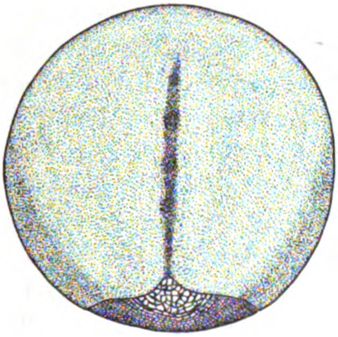

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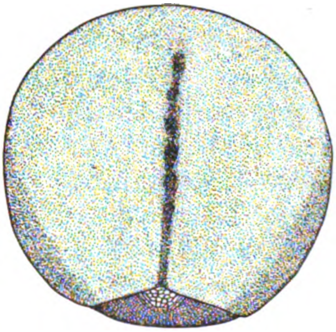

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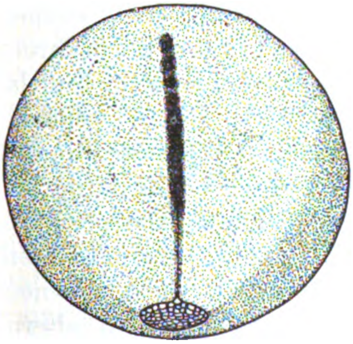

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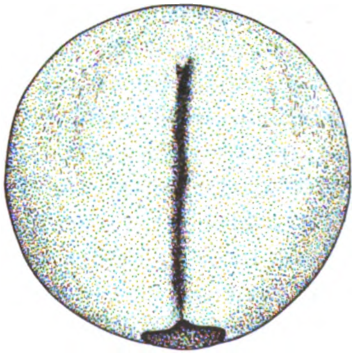

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furrows at the equator on the antero-ventral side of the egg. It usually disappears by the time the neural groove is well established.

In preserved material, the roof of the gastrocoele is considerably paler than the remaining surface of the egg; during the latter part of this stage the neural plate is usually differentiated as a spatulate area extending from the dorsal lip of the blastopore to a little distance in front of the upper pole of the vertical axis, and distinguishable through the greater whiteness of its surface (see especially figs. 223 and 224).

The dorsal lip of the blastopore is not a perfect arc of a circle, but is somewhat incurved on each side of a forward-extending notch in the median line (figs. 138 to 145).

At the time of its first appearance, the neural groove occurs as a distinct furrow extending *from the notch in the dorsal lip of the blastopore* forward in the median line for a distance of about 60 degrees; the anterior half is much broader and deeper than the posterior half (fig. 140). In a slightly later stage, the neural groove has extended to a total length of about 95 degrees but is nowhere so deep as in the anterior half during the preceding stage (fig. 141). It is now a rather shallow groove, narrow in its posterior portion, wider and more broken by occasional deeper depressions or fissures in its middle and anterior parts. These early transverse furrows do not occur at very regular intervals, and are probably only incidental to the process of infolding of the tissues.

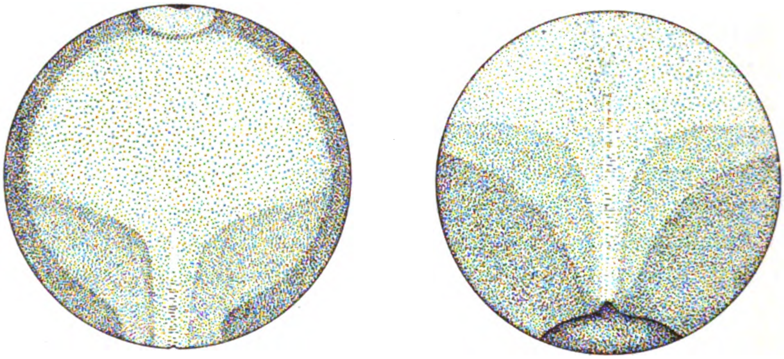
A little later, the neural groove becomes decidedly deeper in its middle portion (fig. 142). The change is not uniform throughout this region, but instead there is a series of three or four large pits or depressions at fairly regular intervals, giving a segmented appearance to the groove. Sometimes this segmented condition is very marked; it has been repeatedly observed in living material. Gradually the segmented region, though less sharply marked becomes more extensive than before (fig. 143); it is best seen in living material viewed by transmitted light, when the neural groove appears made up of a regular succession of alternate light and dark areas. Shortly before the appearance of the neural folds, the neural groove becomes conspicuous in its anterior as well as its middle portion by the broadening and deepening of the former region; at the same time the posterior end becomes fainter (fig. 144).

At the time of the first faint indications of the neural folds, the neural groove is both broad and deep throughout its entire length but especially in its anterior portion (fig. 145). During these later changes in the breadth and depth of the neural groove there has been very little increase in length; at the close of the period considered it has a length of about 105 degrees and extends from the dorsal lip of the blastopore nearly to the upper pole of the vertical axis.

According to Griggs ('10), in Amblystoma the first groove to appear in the median line of the neural plate is not the neural groove, properly speaking; this appears later on the same site. There first appears a 'posterior germinal depression' which does not reach the blastopore; a little later an 'anterior germinal depression' is formed, which is discontinuous with the earlier groove. In a later stage both give place to the neural groove which is a different structure on the same site.

In Cryptobranchus the earliest groove to appear in the median line of the neural plate extends forward without break from the dorsal lip of the blastopore. The marked depression shown in figure 140 probably corresponds to the 'posterior germinal depression' in Amblystoma; the later depression in the anterior portion of the neural groove perhaps corresponds to the 'anterior germinal depression,' but it is at no time sharply separated from the remainder of the groove. The later history of the groove will be given in the following stages and should be consulted in this connection; but it may here be stated that after a careful study of both surface views and serial sections I have come to the conclusion that the differences in the grooves appearing early and late in the median line of the neural plate of Cryptobranchus are differences in degree, not in kind, hence I have used the term 'neural groove' throughout.

In living material the embryo may be viewed by transmitted light. During the early part of this stage (figs. 146 and 147) the broad lateral bands lying in the posterior part of the egg at some distance from the median line are more marked than in the preceding stage. The study of sections shows that they are due to the combined optical effect of the mesoderm and an unusually thick region of the entoderm. The neural groove is particularly translucent. The greatly reduced blastocoele persists in the region of the equator on the antero-ventral side of the egg; the center of its external wall is marked by a pit, the vestige of the fenestra. During the latter part of Stage 12 (fig. 148) the lateral bands are obscured by the thickening of the neural plate; in the central portion of the neural groove there usually appear a series of translucent pits arranged at regular intervals. The pit marking the site of the closing fenestra has disappeared, but there usually remains a translucent area indicating a vestige of the blastocoele; this area is often imperfectly separated from the translucent roof of the gastrocoele.



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Figs. 146 and 147 A living egg of Cryptobranchus allegheniensis in an early neural groove stage, viewed so far as possible by transmitted light. Figure 146 shows the upper hemisphere, figure 147 a postero-dorsal view. × 7.

In this stage it is usually impossible to identify cleavage furrows in the yolk plug, but the stability of the larger cells and the fact that they remain longest exposed afford a means of locating approximately the vegetal pole. We have seen that in the preceding stage the vegetal pole was situated a little above the center of the area of largest macromeres; at the close of Stage 12 these largest macromeres are the only ones exposed, and we may feel quite sure that the vegetal pole lies in their midst and probably very near the center of the greatly diminished yolk plug. The morphological axis of the egg is thus approximately determined by a line passing from the center of the yolk plug through the center of the egg, and at the close of Stage 12 this axis makes an angle of 52 degrees with the vertical—showing that rotation has proceeded 8 degrees further than in the preceding stage (fig. 149).

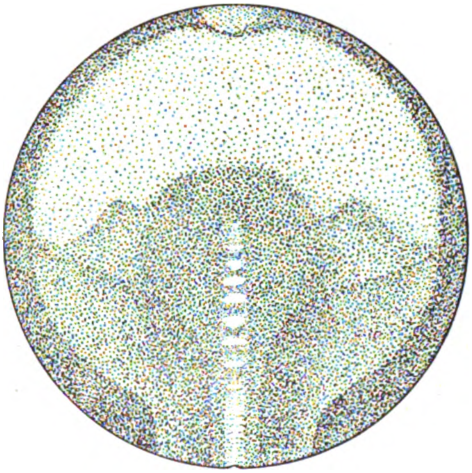
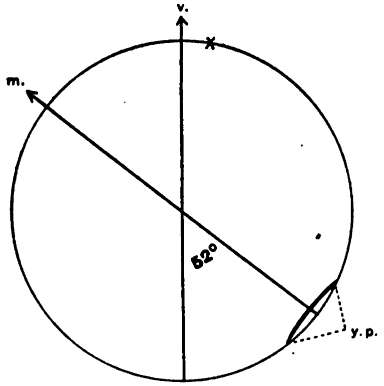


Fig. 148 Upper hemisphere of a living egg of Cryptobranchus allegheniensis viewed so far as possible by transmitted light, shortly before the appearance of the neural folds. × 9.

It will be recalled that the dorsal lip of the blastopore first appears, on the average, 68 degrees above the vegetal pole (fig. 134), and that the ventral lip is first formed about 68 degrees from the vegetal pole on the opposite side (fig. 136). Hence the dorsal and ventral lips are formed respectively at approximately equal distances from the vegetal pole, though the ventral lip is formed much later than the dorsal. We may now compute the amounts of overgrowth of the dorsal and the ventral lips respectively: at the close of Stage 12 the dorsal lip has overgrown the yolk for an average distance of 55 degrees, and the ventral lip has advanced toward it through an arc of about 55 degrees;



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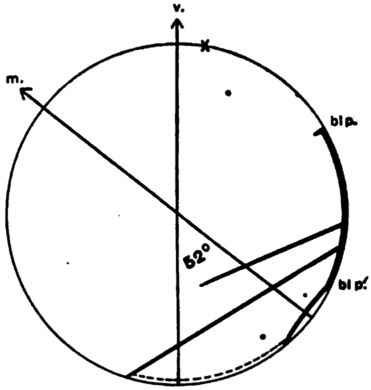

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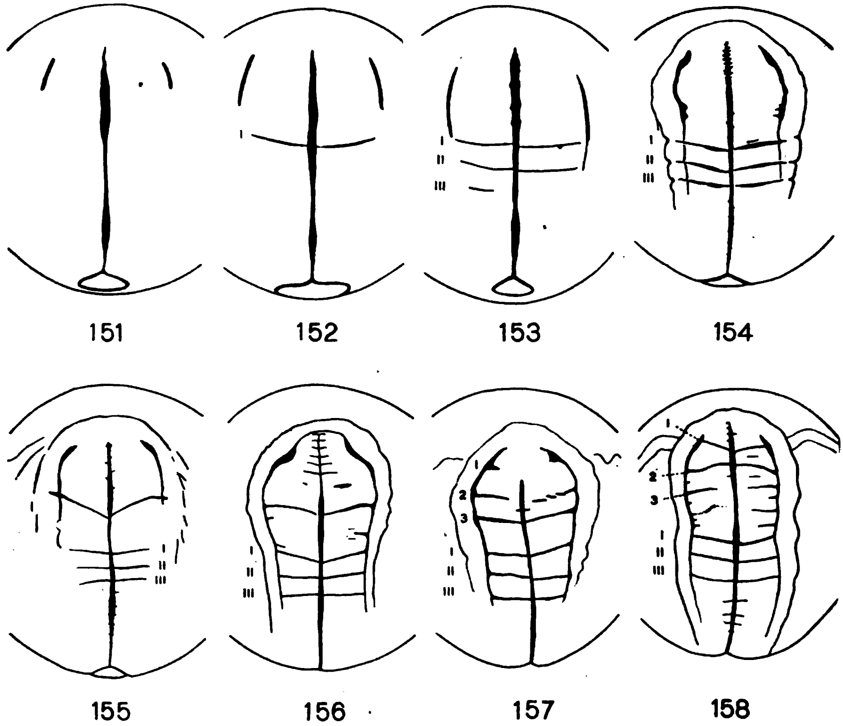
Fig. 149 Diagram of an egg of Cryptobranchus allegheniensis at the close of Stage 12, showing the amount of rotation and the position of the yolk plug; *m*, morphological axis; *v*, vertical axis determined by gravity; *y.p.*, yolk plug. The cross indicates the position of the anterior end of the neural groove.

Fig. 150 Combination of figure 144 with some features of figure 137, showing successive positions of the blastopore; *blp* and *blp′* respectively indicate early and late positions of the dorsal lip of the blastopore. The black band at the right of the figure indicates the amount of overgrowth (55 degrees) of the dorsal lip of the blastopore; the dotted line indicates the amount of overgrowth (55 degrees) of the ventral lip.

that is to say, the distances are approximately equal (fig. 150). By far the greater amount of overgrowth of the dorsal lip occurred during the preceding stage, hence it is clear that during the present stage overgrowth is taking place much more rapidly at the ventral than at the dorsal lip of the blastopore.

Stage 13: (figs. 151 to 164 and 226 to 228). The most conspicuous changes during this stage are those concerned with the formation of the neural folds and the segmentation of the neural plate. The neural folds begin to form about one and one-half days after the appearance of the neural groove. During the progress of this stage the neural groove becomes most conspicuous in an anterior and a posterior portion, separated by a middle region in which it is comparatively faint (see especially figs. 151 to 155).

The early stages in the formation of the neural folds are shown in figures 151 to 158 and need no further description, save to men-



Figs. 151 to 158 Stage 13. A series of embryos of Cryptobranchus allegheniensis showing early stages in the formation of the neural folds and the segmentation of the neural plate. Camera drawings, finished under the binocular, from preserved material. I, II and III indicate the earliest transverse grooves; 1, 2, 3, grooves appearing a little later, numbered consecutively and not in the order of appearance. × 6.

tion that the surface just outside of the neural folds becomes very much roughened and traversed by fissures parallel to the folds, indicating stresses and the rapid shifting of material. During the later part of this stage a pair of less conspicuous transverse folds appear lateral to the anterior end of the neural plate (fig. 158); the significance of these folds has not yet been determined with certainty (but see Stage 15).

The first transverse groove to cross the neural plate is shown in figure 152. A little later, two transverse grooves appear in rapid succession posterior to it. These first three transverse grooves are equidistant, and so distinct that they may readily be seen with the naked eye; since they regularly appear in the same order and position in different embryos, and persist throughout the further history of the open neural plate, they serve as trustworthy landmarks during the following stages. In the figures they are numbered with Roman numerals. By following their history through later stages they have been traced to the region of the medulla oblongata of the adult brain; consequently, at least all that portion of the neural plate in front of Groove III belongs to the cephalic plate.

The early segmentation of the cephalic plate in front of Groove I will now be considered. There first appears a transverse groove dividing this region into two portions of which the posterior is slightly the smaller (figs. 155 and 156); the anterior of these areas is then crossed by two more grooves (figs. 157 and 158), while the posterior area is for the present doubtfully segmented. The smaller transverse grooves occurring in various parts of the cephalic plate are irregular in position and probably are of no segmental value; most of them disappear in later stages. Those grooves in front of Groove I which are regarded as of metameric value are numbered with Arabic numerals, consecutively and without regard to the order of appearance.

The question naturally arises whether these early transverse divisions of the cephalic plate are neural in origin or secondarily produced by the segmentation of the underlying mesoderm. This question has not yet been thoroughly investigated by the study of sections, but the results of a preliminary examination favor the idea that in front of Groove I at least, they are primarily neural structures; the mesoderm, particularly in front of Groove I, is at this time quite thin as compared with the neural plate, and hardly capable of producing the modifications of the latter layer.

Since Grooves I, II, III, etc. (see also Stage 14) are produced in regular order from before backward there is ground for suspicion that they are intimately connected with the formation of the mesoblastic somites. In view of the fact that the segmentation of the region immediately in front of Groove I is late in appearing and seldom clearly expressed (Stage 14), we must be on our guard against a possible discontinuity or difference in kind between the segmentation of the anterior and the posterior regions of the cephalic plate. These points can be settled only by a careful study of sections of eggs that have first been described externally; but from surface views alone we are justified in claiming that we have in the open cephalic plate transverse divisions which may be homologized in different embryos, and which are probably of true metameric value; hence they may be of use in solving the vexed problem of the segmentation of the vertebrate head.

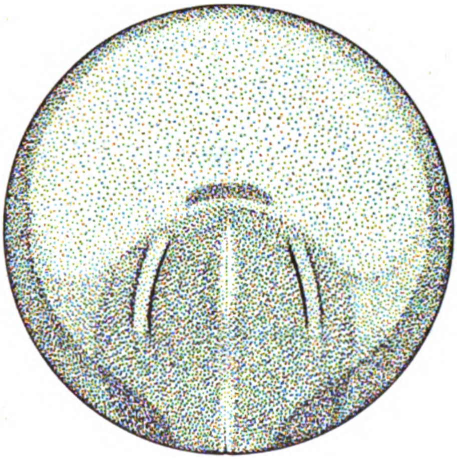


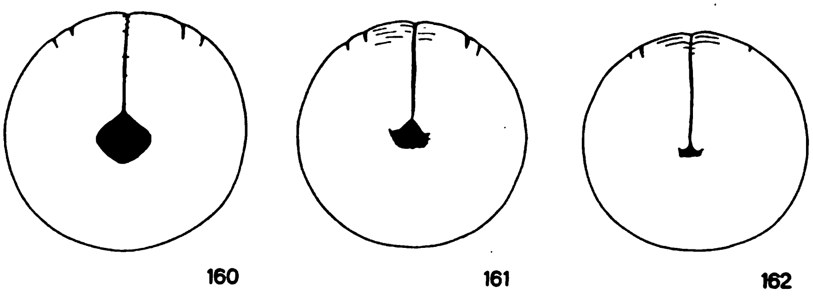
Fig. 159 A living embryo of Cryptobranchus allegheniensis in the early part of Stage 13, viewed in direct sunlight, and so far as possible by transmitted light. From a freehand sketch of the upper hemisphere. × 10.

A pair of depressions just within the neural folds near the anterior end of the cephalic plate probably indicate the anlage of the optic vesicles (cf. Eycleshymer '95; Locy, '95).

Some features of this stage are best brought out by the study of living material; for this purpose embryos have been examined in direct sunlight. As shown in figure 159 a transverse opaque band early appears directly in front of the neural plate in the median region; in position and appearance it reminds one of the ectamnion of the chick (Lillie '08, pp. 138 and 139). The neural folds are conspicuous at an earlier stage in living than in preserved material. In embryos later than the one figured, transverse furrows in the neural plate appear as described in preserved material.

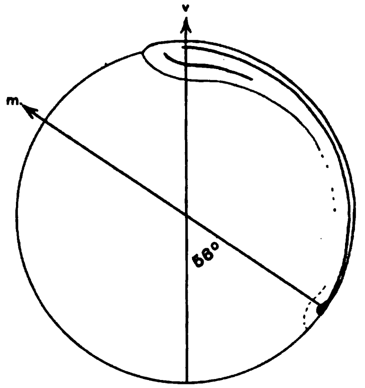
During Stage 13 the blastopore nearly closes, then makes little advance in this respect during the next two stages. Variations in the degree of reduction of the blastopore during these three stages are so great that this structure cannot be used as a character for classifying embryos into stages.

As shown in figures 160 to 162, during Stage 13 the blastopore changes from a diamond shape to that of an anchor; the forward-projecting part is derived through an exaggeration of the notch previously noted in the dorsal lip of the blastopore. The lappets lying on each side of this median notch of the blastopore are continuous with the neural folds; through their apposition the dorsal part of the yolk plug becomes closed over. Thus the extreme posterior end of the embryo is undoubtedly formed by a process of concrescence. As shown in later stages, the ventral part of the blastopore becomes reduced to a transverse slit (figs. 177 and 178); during this process the yolk plug usually becomes entirely withdrawn into the egg, but a small mass of yolk sometimes persists at the surface. The late history of the blastopore is much the same in Cryptobranchus japonicus, as described by Ishikawa ('08).



Figs. 160 to 162 A series of embryos of Cryptobranchus allegheniensis in Stage 13, showing changes in the size and form of the late blastopore. Camera drawings from preserved material. × 5.

At the close of Stage 13 the neural grove has reached a length of about 124 degrees; its anterior end usually lies quite accurately at the upper vertical pole, while the neural folds extend about 16 degrees in front of it. We have seen that the closing blastopore marks the approximate position of the vegetal pole; this pole has now rotated a total distance of 56 degrees from the vertical axis.



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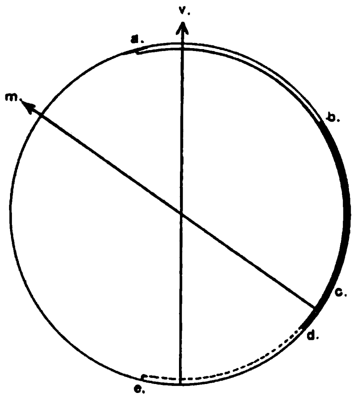

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Fig. 163 Diagram of an embryo of Cryptobranchus alleghenienais at the close of Stage 13, showing the position of the neural plate and neural folds with reference to the morphological and the vertical axes; *m*, morphological axis; *v*, vertical axis.

Fig. 164 Diagram showing the position of the embryonic body of Cryptobranchus alleghenieneis, and illustrating some features of embryo-formation; *a* to *b* (72 degrees), portion of the embryo formed in situ; *b* to *c* (60 degrees), portion formed by overgrowth of the dorsal lip of the blastopore, with the possibility of concrescence; *c* to *d* (roughly estimated at 16 degrees), portion undoubtedly formed by concrescence; *d* to *e* (60 degrees), distance traveled by the ventral lip of the blastopore. Other lettering as in the preceding figure.

We now have sufficient data for a statement of the position of the embryonic body on the egg, and for pointing out certain features of its mode of formation (figs. 163 and 164). About 72 degrees of the anterior end of the embryo is formed in situ. About 60 degrees is formed in connection with the overgrowth of the dorsal lip of the blastopore; in this case there is the possibility of concrescence through the apposition of material on each side of the median notch, which may be shifted toward the median line during the process of overgrowth. This point can be definitely settled only by experiment; but in the absence of experimental data we can say that there is no positive evidence of such a process taking place, while certain considerations weigh against it. For in certain observed cases rapid shifting of material is accompanied by a roughening of the surface with the formation of parallel fissures, as in the region just outside of the neural folds during their formation and progress toward the median line. There is an entire absence of any such feature in the dorsal lip of the blastopore.

A region at the posterior end of the embryo, which is roughly estimated at 16 degrees, is formed through the concrescence of the lateral and ventral lips of the blastopore. A part of this material has been brought through a distance of 60 degrees by the overgrowth of the ventral lip of the blastopore; it will be observed that this distance equals that of the overgrowth of the dorsal lip of the blastopore.

At the close of Stage 13, when the embryonic body is for the first time clearly indicated, it has a total length of about 148 degrees. The posterior end is formed around the vegetal pole; the anterior end lies about 40 degrees from the animal pole. Hence the statement made in Part I (Smith '12) to the effect that the axis of polarity of the late ovarian egg defines the principal axis of the embryo is not quite accurate; but the embryo is formed almost wholly in a hemisphere of the egg lying to one side of the axis of polarity. A review of its history shows that the embryo is formed almost entirely out of material derived from a band of cells lying in the equatorial region of the late blastula and early gastrula, and that this band of cells is narrow on the ventral, broad on the dorsal side of the egg (figs. 116 and 123).

Goodale ('11), after reviewing the literature of the subject in connection with his own work on Spelerpes, concluded that

> The amphibian embryo develops almost entirely in a vertical half of the egg, the tail appearing near the lower pole, while the anterior end of the body develops in greater or less degree in the upper hemisphere,

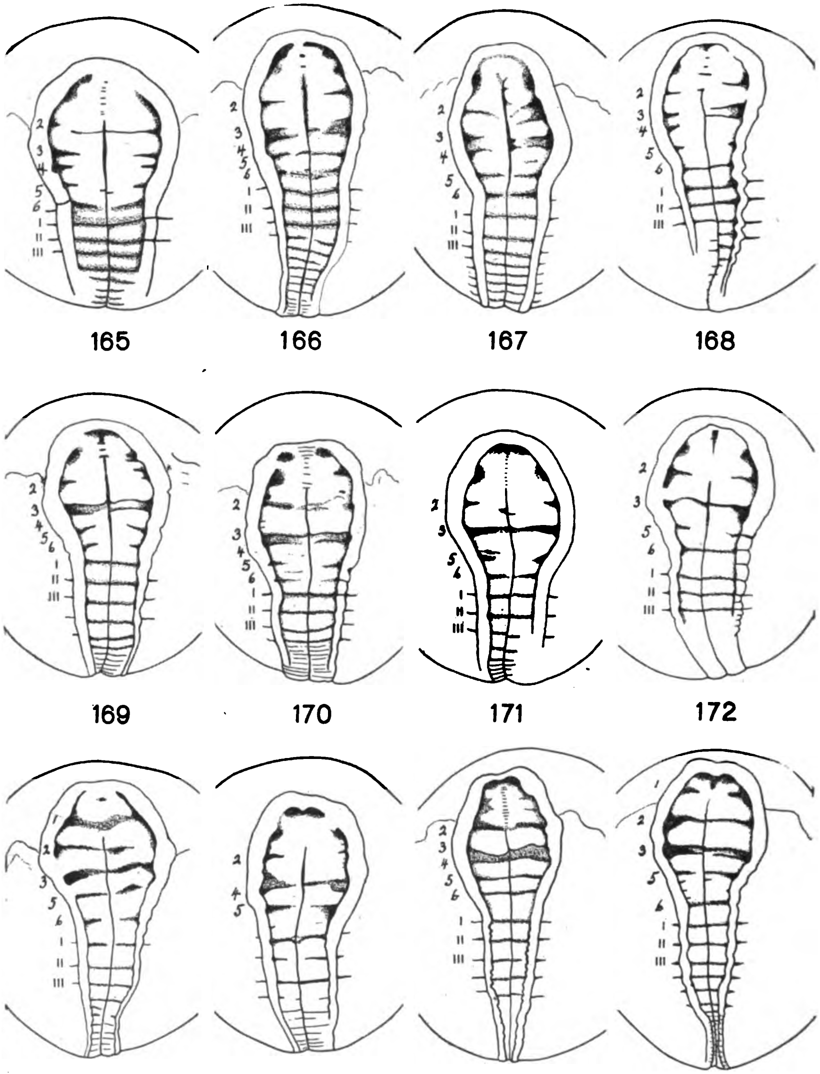
depending upon the particular species. The position of the head of the embryo seems correlated with the length of the embryo, so that the longer the embryo, the higher up on the egg it develops.

The terms 'upper' and 'lower' are evidently here used in the sense of animal and vegetal, that is to say, with reference to those points on the surface of the egg which were in the vertical axis of the egg before it commenced to rotate; therefore the results obtained with Cryptobranchus fall in line with the general statement quoted. The results of Goodale on Spelerpes and my own work on Cryptobranchus agree closely in locating the posterior end of the embryo at the vegetal pole; it is worthy of note that this conclusion was reached independently and by entirely different methods.

Stage 14: (figs. 165 to 179 and 229 to 232). This stage is reached about one day later than the beginning of Stage 13. Since at this time scarcely any two embryos agree in the rate of development of homologous regions of the body, it is impossible in this stage to make a close classification. In marking off this stage from the one following, the principal character considered is the approach of the neural folds toward the median line.

Figures 165 to 176 represent twelve embryos that illustrate the principal changes in the antero-dorsal region during this stage. It will be seen that there is a progressive addition of transverse grooves posterior to the three that first appeared. In front of Groove I the cephalic plate is traversed primarily by six grooves; of these Groove 1, which was noted in the preceding stage, has a very transitory existence and in most cases is lost in Stage 14; likewise the median portion of Groove 2 has often disappeared. Moreover in this or the following stage Groove 4 disappears, following a marked depression and perhaps submergence of the segment between it and Groove 3.

A significant relation exists between Grooves I, II, III, etc., and the intersomitic grooves which now appear just outside the neural folds; by an inspection of figures 165 to 176 it will be seen that in all cases these are in direct apposition. Since the mesoblastic somites are the most characteristically segmented structures of the vertebrate body, it follows that the true segmental units of



165 166 167 168

169 170 171 172

173 174 175 176

Figs. 165 to 176 Antero-dorsal views of embryos of Cryptobranchus allegheniensis in Stage 14, showing especially the segmentation of the neural plate. Camera drawings finished under the binocular, from preserved material. × 6.

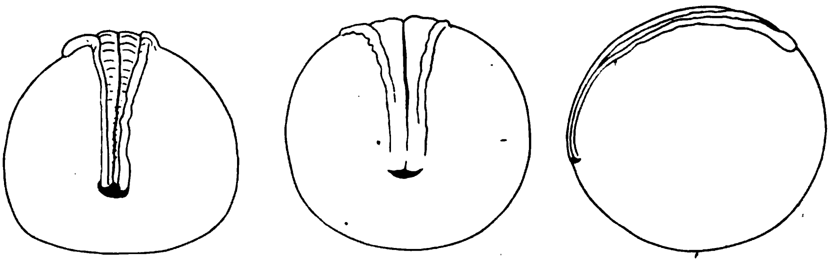
The earliest transverse grooves to cross the neural plate are numbered with Roman numerals in the order of appearance; in front of Groove I the transverse grooves are numbered with Arabic numerals consecutively without regard to the order of appearance. Figure 166 is from the embryo photographed for figures 229 and 230; figure 176 is from the embryo photographed for figures 231 and 232.

the open neural plate, in this region at least, are the divisions between grooves—that is, the ridges rather than the depressions, for the former are in line with the body somites. If, as appears likely, there is continuity between the structures of the anterior and posterior regions of the cephalic plate, then the rule may be extended to include the entire neural plate. As thus defined, there are seven segments—'neuromeres'—in front of Groove I; posterior to this groove an undetermined number of segments also belong to the head.

In the early stages of the formation of the neural folds transverse grooves are sometimes found in them, continuous with the transverse grooves of the neural plate (see especially figs. 154, 165 and 172). In such cases the neural fold is marked by an outer as well as an inner notch, both in line with the transverse furrow of the neural plate. This condition is only temporary and apparently it is transitional to a later phase in which the inner notch grows at the expense of the outer one, until an outer convexity of the fold appears opposite the inner concavity (see especially fig. 168). In the region of the body somites these outward flexures thus lie in line with the intersomitic grooves as well as with the transverse grooves of the neural plate. This condition is seldom so well expressed as in the embryo shown in figure 168; the convolutions of the neural folds are often irregular and bear no definite relation to the segments. But it is fairly certain that in all cases where the neural folds are well upraised and flexures occur which are segmentally arranged, the outward flexures lie opposite the transverse furrows and not opposite the ridges between them. Moreover, in sagittal sections the transverse grooves on the external surface of the neural plate are found to correspond to ridges on the internal surface.

Those who have described segmental structures in the neural folds or closed neural tube have, as a rule, accepted Orr's ('87) definition of the segmental units or neuromeres as *outward* flexures of the neural folds. But if the above considerations be well founded, the true segments are to be sought rather in the segments between the transverse grooves of the neural plate, and in the *inward* flexures of the neural folds. In other words neuromeres are the transverse ridges on the inside rather than on the outside of the brain. To a limited extent this view coincides with that of Kupffer ('85 to '93), who maintained that the true neuromeres are the transverse divisions of the open neural plate rather than the later appearing structures in the neural folds.

Most of the features of this stage thus far described have been observed in living as well as in preserved material. The literature on the early development of the central nervous system has recently been reviewed by Griggs ('10); a more comprehensive survey of the earlier work on the segmentation of the vertebrate head is given by Locy ('95). Ishikawa ('08) has described segmental divisions in the open neural plate of Cryptobranchus japonicus.



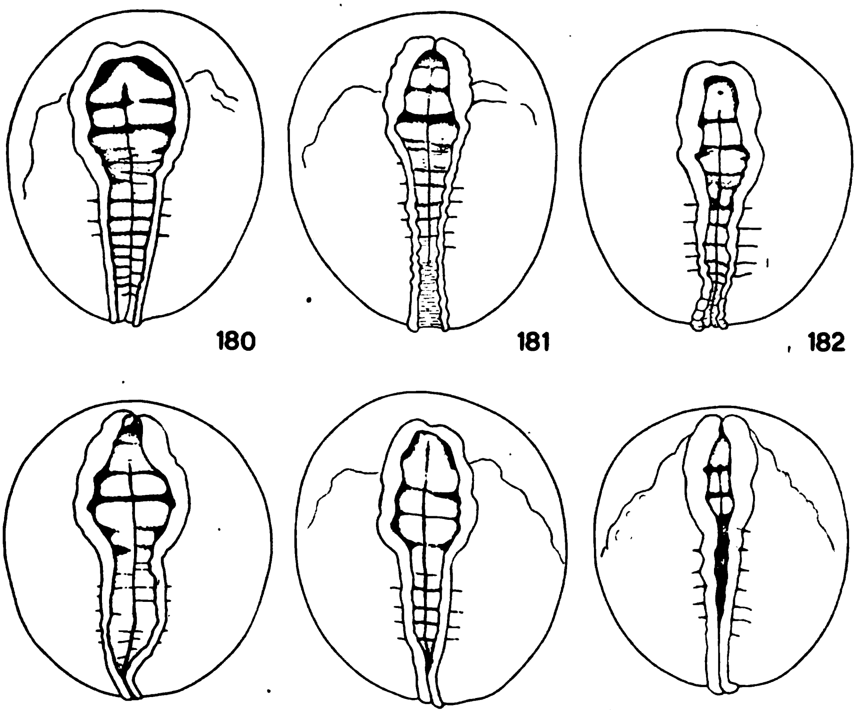
177 178 179

Figs. 177 to 179 Camera outlines of embryos of Cryptobranchus alleghenienis in Stage 14, drawn from preserved material. × 5.

Figs. 177 and 178 Posterior views showing late blastopore.

Fig. 179 Lateral view showing position of the embryonic body at the close of Stage 14. The egg is shown in its natural position with respect to the vertical axis, which passes in the plane of the paper parallel to its lateral margins. The embryo proper has a total length of about 155 degrees. This figure and figure 166 are drawn from the same egg.

During this stage, if not already in the preceding stage, the anterior or dorsal part of the blastopore becomes closed over, while the ventral part persists as a transverse crescentic slit (figs. 177 and 178). At the close of Stage 14 the embryo has increased slightly in length (fig. 179); it now extends over about 155 degrees of the surface of the egg. This increase in length



180 181 182

183 184 185

Figs. 180 to 185 Antero-dorsal views of embryos of Cryptobranchus allegheniensis in Stage 15. Camera drawings finished under binocular, from preserved material. Figure 182 is drawn from the embryo photographed for figure 234; figure 184 is drawn from the embryo photographed for figure 233. × 6.

of the embryo involves a noticeable increase in the antero-posterior dimension of some of the neuromeres.

Stage 15: (figs. 180 to 189; 233 and 234). This stage is reached about eighteen hours later than the beginning of the preceding stage.

In the following account, each neuromere is designated by the number of the groove bounding it on the posterior side. Neuromeres 1 and 2 have usually coalesced; neuromere 4 disappears during this, if not in the preceding stage. More definite swellings now occur in neuromeres 1, 2, 3 and 5; the region between Grooves 5 and I is less clearly segmented and is usually somewhat depressed. The outlines of the neural folds in the head region now suggest the definitive primary divisions (forebrain, midbrain and hindbrain) of the embryonic brain.

The various structures of the neural plate have not yet been followed into the definitive divisions of the embryonic and adult brain; but the preliminary examination of some later embryos dissected by splitting them in the median line with a razor shows that the transverse divisions in the neural plate persist for some time after the closure of the neural folds. Neuromeres in the closed neural tube are also often apparent from the surface. Hence it is easy to judge approximately concerning the fate of individual neuromeres of the cephalic plate, but to avoid possible error it seems best to defer a definite statement until the internal history of the brain has been more carefully studied.

The pair of folds which in the preceding stages extended transversely on each side of the cephalic plate now slant backward (see especially fig. 185); the appearance, particularly in living material, suggests that they are in some way concerned with the origin of the vascular bands which in later stages extend along each side of the yolk sac and give rise to the omphalomesenteric or vitelline veins (fig. 192).

The transverse opacity in front of the neural plate is conspicuous in living material viewed by transmitted light (fig. 186), but is not apparent in surface views of preserved material.

The anterior part of the blastopore is now normally closed over, and the posterior or ventral part is reduced to a transverse slit (figs. 188 and 189). Apparently the middle portion of this transverse slit never becomes completely closed, but in later stages persists as the anal or cloacal opening. The embryonic body has elongated so that it now extends over about half the circumference of the egg (fig. 187).

For the study of transverse divisions in the open neural plate, Necturus is not nearly so favorable as Cryptobranchus. In Necturus the blastopore (figs. 268 to 279) closes much earlier than in Cryptobranchus. Moreover in Necturus the closure of the blastopore is often practically complete; in many specimens preserved at the time of the closure of the neural folds, scarcely more than a vestige of the blastopore is visible from the surface.

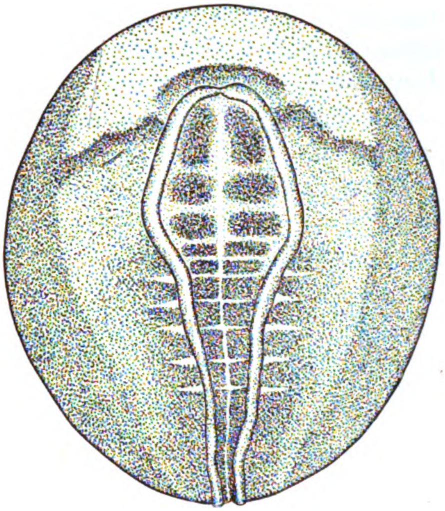


Fig. 186 Antero-dorsal view of a living embryo of Cryptobranchus allegeheniensis in Stage 15, viewed mainly by transmitted light. From a freehand sketch. × 10.

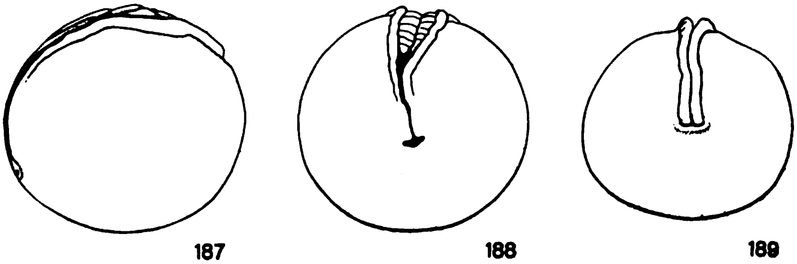


Fig. 187 Lateral view of an embryo of Cryptobranchus allegheniensis in Stage 15, showing the position of the embryonic body. The egg is shown in its natural position with respect to the vertical axis which passes in the plane of the paper parallel to its lateral margins. Camera drawing from preserved material. × 5.

Figs. 188 and 189 Posterior views of embryos of Cryptobranchus allegheniensis in Stage 10, showing the form of the late blastopore. Camera drawings from preserved material. × 5.

Figs. 183, 187 and 188 All drawn from the same embryo.

Figs. 185 and 189 Drawn from the same embryo.

In these later stages, the blastopore is doubtless often indistinguishable in living material (figs. 274 to 276).

A triradiate form of the blastopore is not so frequently found in Necturus; in Cryptobranchus japonicus (Ishikawa '08), it often occurs. A general resemblance may be noted between the blastopore of the urodeles cited and that of the dipnoans (Ceratodus, Semon '01; Protopterus and Lepidosiren, Kerr '09).

B. Summary

Gastrulation involves a combination of the processes of invagination or emboly, and overgrowth or epiboly.

During gastrulation the roof of the segmentation cavity becomes very thin, and is bounded superficially by a sharp furrow, the 'septal furrow' of Ishikawa.

On account of the translucent character of certain parts of the egg, many of the internal changes concerned with gastrulation can be followed quite satisfactorily in living material.

For some time after the beginning of gastrulation, the vegetal pole may be located through the intersection of the first and second cleavage furrows.

During gastrulation and the formation of the neural groove and neural folds the egg rotates on an axis at right angles to the median plane so as to bring the morphological axis at an angle of 56 degrees from the vertical.

The dorsal lip of the blastopore is formed about 68 degrees above the vegetal pole; the ventral lip is formed much later at an equal distance on the other side of the vegetal pole. Since the closing blastopore lies approximately at the vegetal pole, overgrowth proceeds through equal distances on the dorsal and the ventral sides of the egg. During the early part of gastrulation, before the ventral lip is formed, overgrowth takes place rapidly and extensively at the dorsal lip; after the blastopore has become a complete circle, overgrowth takes place very slowly at the dorsal lip, very rapidly at the ventral lip.

Not until after the neural folds are well formed is the yolk plug completely overgrown; as compared with Necturus and most amphibian eggs the blastopore is very late in closing. In late stages the blastopore has the form of an anchor or an inverted *T*; the posterior transverse portion remains longest as an open slit, and the center of this transverse portion never completely closes but persists as the anal or cloacal aperture.

The posterior end of the embryo forms approximately at the vegetal pole. At the time when the neural folds are first formed the embryo has a total length of about 148 degrees, hence its anterior end does not reach the animal pole. About 72 degrees of the anterior end of the embryo (nearly half its total length) is formed in situ; about 60 degrees posterior to this is formed by overgrowth with the possibility of concrescence. Only a very small part at the posterior end, perhaps 16 degrees, is formed by the meeting of the lateral and ventral lips of the blastopore; this part is undoubtedly formed by concrescence.

From the time of its first appearance the neural groove is continuous with a median notch in the dorsal lip of the blastopore. There is evidence that the neural groove early acquires a segmented structure.

Transverse grooves, definite in number and location, cross the neural plate, dividing it into true segments or neuromeres. In the region of the mesoblastic somites the transverse grooves of the neural plate are in line with the intersomitic grooves, and the neuromeres are in line with the somites. Segmental flexures of the neural folds sometimes occur; in these cases the outward flexures of the neural folds are in line with the transverse grooves, and the inward flexures are in line with the neuromeres.

At the time of the closure of the neural folds, the embryo has increased in length so that it extends over about one-half of the circumference of the egg.

IX. DEVELOPMENT AFTER THE CLOSURE OF THE NEURAL FOLDS

A. Description by stages, to the time of hatching

Most of the important features of the later external development are sufficiently illustrated by the photographs. Only a brief account is here necessary and this will deal principally with observations on living material. Some comparisons with Necturus have been given in a previous paper (Smith '11 a). Late stages of Cryptobranchus japonicus have been figured by Ishikawa ('04 and '08) and de Lange ('07).

Stage 16: (*figs. 235 to 237*). This stage is reached about eighteen hours later than the beginning of Stage 15. It is characterized by closed neural folds which are still more or less separated by a median groove. Ganglionic ridges are forming at the sides of the brain. The blastopore is no longer a transverse slit, but a small round orifice which probably represents the definitive cloacal opening. Up to this time the great majority of the eggs have retained the 'vitelline membrane.' During Stage 16 or slightly later this covering usually becomes ruptured as a consequence of the growth of the embryo and is finally cast off.

During the gastrula and open neural groove stages, careful observations have been made to test the presence of cilia on the ectoderm, with absolutely negative results. Currents of water produced by ciliary motion may be detected through the movements of yolk particles within the vitelline membrane when this is present, or by means of powdered carmine added to the water in cases where the vitelline membrane has been shed. At the time when the neural folds are closing, cilia are present on the sides of the body and the ventral surface of the yolk sac, but are absent from the neural folds. The general direction of the ciliary currents is toward the posterior end of the body.

Stage 17: (*figs. 238 to 242*). This stage is reached about a day later than Stage 16. The neural folds are definitely closed and the head well upraised. The optic vesicles are indicated by slight paired expansions of the anterior part of the brain. In some embryos the anlage of the pronephros is apparent through an elevation of the overlying ectoderm. Cilia are absent from the dorsal surface above the neural tube but are quite generally present elsewhere and are particularly strong or numerous on the dorsal surface of the body, lateral to the neural tube. In general the beat of the cilia along the sides and ventral surface of the body is backward.

The embryo is still erect (i.e., with the dorsal surface uppermost). In this position it has been observed, in many cases, to rotate slowly on a vertical axis. To test the direction of rotation a large number of embryos were placed separately in watch glasses and individual records made. Out of sixteen embryos that showed rotation, only two moved in a clockwise direction, the other fourteen in an anti-clockwise direction. The rotation is, of course, caused by the cilia. The direction of rotation in this stage can hardly be explained as the result of a tendency for the embryos to lean to one side oftener than to the other, for, as will be shown in a later stage, the facts are otherwise. Possibly more extended observations would show more equality in the results; or there may be a uniform asymmetry in the distribution, or in the rate or direction of beating, of the cilia of the ventral surface of the yolk sac.

Stage 18: (figs. 243 and 244). This stage is reached about twenty-four hours after the beginning of Stage 17. It is characterized by a prominent outstanding head with marked cephalic flexure and distinct optic vesicles, and by the presence of the pronephros and the first definite indications of the budding tail. During the latter part of this stage the mandibular arch is usually recognizable.

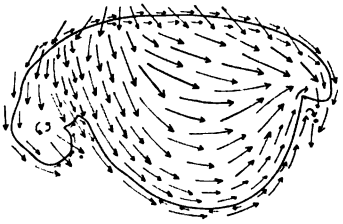
Patches of cilia are now distributed over the entire surface; the beat of the cilia is in general backward and the currents are much the same as figured in the next stage (fig. 190).

During this stage the embryo topples over from its erect position so as to fall to one side, on which it lies throughout several succeeding stages until spontaneous movements enable it to change its position. An incidental result of this position is to bring a larger area of the ciliated surface into contact with the substratum; as a consequence of this and of the stronger ciliation, rotation of the embryo is now of more marked occurrence. The most rapid motion observed was performed by an embryo that completed a rotation in just two minutes.

The functional value of the ciliary motion is at least two-fold: (1) it bathes the surface of the embryo with currents of water which are subservient to respiration; and (2) rotation of the embryo, when it occurs, serves to prevent adhesion of the embryo to the envelope with consequent abnormalities.

The ciliation and rotation of the frog embryo have been described by various writers, notably Assheton ('96). Piersol ('09) has described rotation in the embryo of Plethodon.

Stage 19: (figs. 190 and 245 to 248). This stage begins about twenty-four hours later than Stage 18. It is characterized by from two to three distinct gill invaginations, a budding tail, a very marked outward expression of the pronephros (see especially figs. 245 and 246), and beginning lateral vascular bands, the anlage of the vitelline veins (see especially fig. 245). In addition to the cephalic flexure there is a slight cervical flexure which reaches its maximum in this stage. About sixteen to twenty mesoblastic somites are apparent in surface views.



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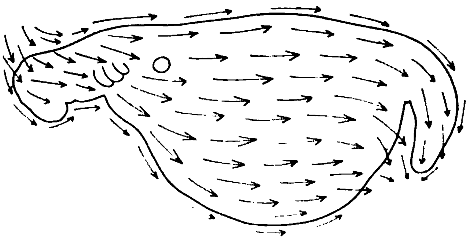

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Fig. 190 Diagram of an embryo of Cryptobranchus allegheniensis in Stage 19, showing the direction of the water currents produced by cilia.

Fig. 191 Same as figure 190, for Stage 21.

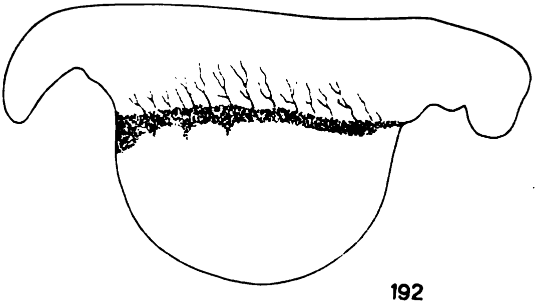
In the living embryo, the lateral vascular bands are conspicuous structures, for they are pink with blood; but they are not yet differentiated into true veins. Overlying the upper part of the yolk sac, they extend from the heart region longitudinally on each side of the body and meet posteriorly a little below the tail. During this stage and the stages immediately following, they shift slowly toward the ventral surface. There is considerable variation in the position of this band in embryos that are otherwise in the same stage. A similar vascular area has been figured for Cryptobranchus japonicus by de Lange ('07).

In this stage practically all embryos are found lying on either the right or the left side. To determine the relative number of cases of each, one hundred and nine embryos, taken at random from several different spawnings, were examined; fifty were found to lie upon the right side, fifty-nine upon the left side. These numbers are approximately equal, hence the occurrence follows the laws of chance.

Rotation of the embryo now occurs in a greater proportion of cases than in the preceding stage, though of the embryos studied hardly more than one in ten has been observed to rotate. It is noticeable that in some cases the direction of rotation is clockwise, in other cases anti-clockwise. In eleven embryos that showed rotation eight moved in an anti-clockwise direction; all these were lying on the left side. Three embryos rotated in a clockwise direction; of these two were lying on the right side, one on the left side. Hence as a general rule the direction of rotation is correlated with the position of the body. The actual direction is not what one would expect if the beat of the cilia were directly backward in all parts of the body; but by an inspection of figure 190 it will be seen that in the head region the beat is ventrad, in the posterior region dorsad. The ciliary currents are alike on the two sides of the body.

Stage 20: (figs. 192 and 249 to 251). This stage is reached about two days later than the beginning of Stage 19. Through the loss of the cervical flexure the head is now brought more nearly in line with the body, but on the other hand there is an increase in the cephalic flexure. Five gill invaginations are usually visible, the two posterior ones being sometimes indistinct. There are distinct nasal pits. The roof of the medulla is becoming thin and transparent. From twenty-five to thirty mesoblastic somites are apparent in surface views. The tail undergoes a decided increase in size, and is slightly flexed ventrally. The front limb anlagen appear during the latter part of this stage.

The lateral vascular bands are not yet differentiated into veins, but during the latter part of this stage some small veins have been observed, in living material, extending vertically from the vascular bands for a short distance above them (fig. 192). No



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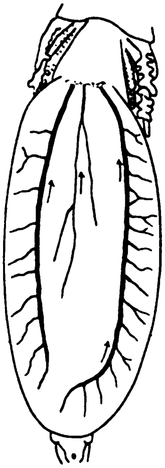

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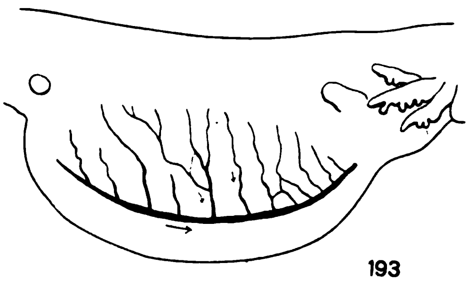

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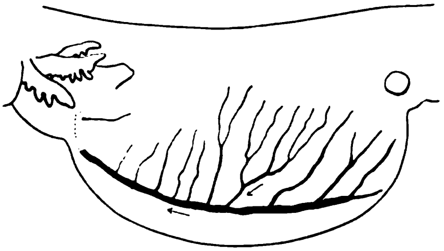

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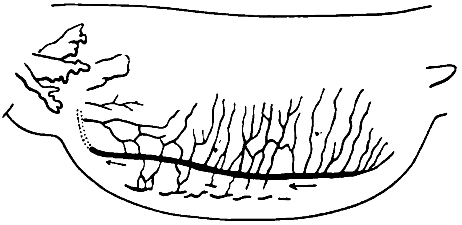

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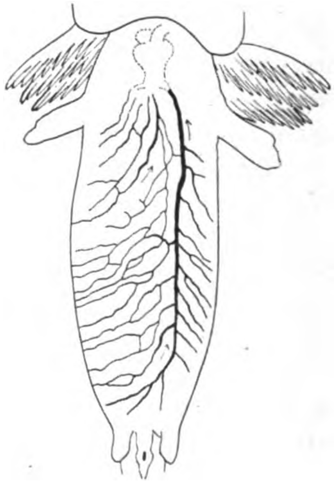

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Figs. 192 to 197 Development of the vitelline veins of Cryptobranchus allegheniensis. All the figures are from living material and, with the exception of the veins in figure 192, are drawn with the aid of a camera. × 5.

Fig. 192 Stage 20. The stippled area indicates the lateral vascular band. The right and left sides of the embryo are practically alike.

Figs. 193 and 194 Right and left views of an embryo in Stage 22.

Fig. 195 Lateral, slightly ventral, view of an embryo nearly ready to hatch.

Fig. 196 Ventral view of an embryo nearly ready to hatch.

Fig. 197 Ventral view of a larva about ten days after hatching.

movement of the blood has been observed in this stage. The embryos are ciliated, and undergo rotation, much as in the preceding stage. Muscular movements do not ordinarily occur, but have been observed when the embryos were placed in fixing fluids.

Stage 21: (figs. 191 and 252 to 255). This stage is reached about four days later than the beginning of Stage 20. It is characterized by the presence of three budding external gills, and small front limb rudiments. The tail is longer, and has a more decided ventral flexure, than in the preceding stage.

The dorsal surface of the embryo is now for the first time slightly pigmented. The pigmentation begins in that portion of the ectoderm overlying the nervous system, and gradually extends downwards over the sides of the body. During this and the following stages (McGregor '97), the pigment cells in the region of the mesoblastic somites are grouped metamerically.

Numerous veins now branch off dorsally from the lateral vascular bands. In the bands themselves, the two main trunks of the omphalo-mesenteric or vitelline veins, one on each side of the yolk sac, are being differentiated, but they rarely become complete before the next stage. In general the differentiation of the vitelline veins has gone further on the side of the body that happens to be uppermost. During the latter part of this stage the heart is pulsating regularly, with about twenty-five to forty beats per minute; the blood sometimes surges back and forth in the principal veins overlying the yolk sac, with a slight excess in the forward movement, but the vitelline circulation is not yet completely established.

The distribution of cilia remains much the same as in the preceding two stages, but the ciliary currents move more uniformly toward the posterior end of the body (fig. 191).

Spontaneous muscular movements, consisting chiefly of a bending of the body laterally into a *U* shape, now occur, but the embryo is as yet unable to turn over.

Stage 22: (figs. 193, 194 and 256 to 259). This stage begins about five days later than Stage 21; its most distinctive characteristic is that the external gills have rudimentary branches and are pink with blood. The tail is rapidly increasing in size and beginning to straighten out. The front limb rudiments now take the form of conspicuous outstanding lobes, the distal ends not yet divided into digits. Toward the close of this stage the rudiments of the hind limbs appear. The entire dorsal surface of the body is well sprinkled with pigment cells; the eyes are becoming deeply pigmented. The lateral line system is well developed. During this stage occurs the rapid formation of the gular folds.

The lateral vascular bands no longer appear as such, but on their site are differentiated the two main trunks of the vitelline veins (figs. 193 and 194). As compared with the earliest position of the lateral vascular bands, the vitelline veins lie considerably nearer the ventral surface of the yolk sac. While the vitelline system of veins is primarily a paired one, almost from the beginning one side is usually found better developed than the other. The heart now contracts at the rate of about forty to sixty beats per minute, and the blood pulsates regularly through the vitelline veins.

The cilia are especially well developed on the external gills; here the ciliary currents are strongest. Spontaneous muscular movements now occur at frequent intervals. The movements consist of jerking the head from side to side; wriggling; reversal of the laterally curved position of the body by turning over; swimming movements by means of which the embryo butts against the envelope; and swimming in a circle. The functional value of these movements seems to be to afford exercise for the developing muscles. Embryos removed from the capsules at this stage make practically the same movements; they are unable to progress in a straight line and are incapable of prolonged swimming movements.

During the later stages of development before hatching, the water in which the embryos are kept has a pronounced 'fishy' odor.

Stage 23: (figs. 195, 196, 260 and 261). The limits of this stage are fixed to include the time of hatching. In a given lot of embryos hatching does not take place all at once, but extends over a period of about a week, with a corresponding variation in the degree of development of different individuals at the time of escape from the envelopes. On the average, hatching occurs about two weeks later than the beginning of Stage 22, and about six weeks after fertilization. Previous to the hatching period, the envelopes become much softened and considerably enlarged by the absorption of water, making room for the growing embryo. The latter usually escapes by pushing ('worming') its way through the envelope, leaving a small round hole; in some cases it bursts the envelope by means of wriggling movements.

The newly hatched larva measures about 23 to 25 mm. in length. Very noticeable is the retention of a large yolk sac with conspicuous bright-red vitelline veins; the bushy external gills are pink with blood. In proportion to size of body, the tail is much larger than in the adult. The dorsal surface of the body and the sides of the tail are well pigmented, but in general the larva of Cryptobranchus, like that of Necturus, is pale as compared with amphibian larvae that develop from a pigmented egg exposed to the light. The ventral surface is lacking in pigment, leaving the abdominal region yellow from the presence of yolk, and the throat region transparent. The heart can be readily observed without dissection. The anterior limb rudiment is provided with two digits. In most specimens the body somites are plainly visible, but they do not show well in the photographs. On account of its large size, graceful outlines and bright colors, the newly-hatched larva is a striking and beautiful object.

In the resting position, the larva lies on its side, turning occasionally from one side to the other. The newly hatched larva is able to swim rapidly in a straight line for a short distance, using the tail as a propeller. The larvae avoid the light, and are positively rheotactic.

The vitelline veins (figs. 195, 196 and 261) have shifted further toward the mid-ventral line; on one side of the body these veins are well developed, on the other side they show arrest of development with signs of atrophy. The heart now beats about sixty to seventy times per minute.

Aëration of the blood is afforded, not only by the external gills, but by the capillaries lying close to the surface over all the body. On account of its great exposed surface, the tail may be of especial importance as a respiratory organ.

As in the preceding stage, cilia persist over the entire surface of the body, and the ciliary currents are strongest in the vicinity of the gills.

B. Larval development, and the metamorphosis

The changes in the form of the body, and the gradual increase in size, during the first year of larval development, are shown by the photographs (figs. 262 to 267).

Year-old larvae reared in the laboratory reach a length of about 5 to 7 cm.; three two-year-old specimens reared in the laboratory measured respectively, after preservation in alcohol, 7 cm., 8 cm. and 9.5 cm. Near the close of the second summer these latter specimens lost their external gills. The few specimens with external gills taken in August from their natural environment (they were found under small flat stones in shallow water) measured as follows: 6.4 cm., 6.8 cm., 7.0 cm., 7.3 cm., 7.7 cm., 12.0 cm., 12.3 cm. It will be noticed that these specimens sort into two lots, one lot containing those with body lengths ranging between 6 and 8 cm., the other lot containing specimens approximately 12 cm. in length. Though this data is rather meager, the rather considerable gap between the two lots suggests that we are dealing with larvae of the first and second summers, respectively. In comparing the larger larvae taken from their natural environment with the two-year-old specimens reared in the laboratory, allowance must be made for the fact that the latter were measured after being shrunken by preservation in alcohol; moreover the body form of the laboratory specimens seems to be shorter and stouter than the normal. Specimens with a body length of 14 cm. and more, taken from their natural environment, have invariably lost their external gills. The combined evidence from specimens reared in the laboratory and those taken from their natural habitat indicates that the metamorphosis occurs at the end of the second year.

At the time of hatching, the embryo retains a supply of yolk sufficient to last it for several months; the mouth is still quite ventrally situated. So far as its method of nutrition is concerned, during this period the young Cryptobranchus is an embryo rather than a larva. Gradually the yolk disappears, and the mouth assumes a terminal position. Specimens reared in the laboratory begin to take food about two to four months after hatching; they must be fed individually with bits of scraped beef. No notice is ordinarily taken of the food unless it is moved about immediately in front of the animal and preferably a little to one side of the mouth. Some of the specimens take food more readily, and grow more rapidly, than others. One lot of larvae, reared in the laboratory, ate young frog tadpoles. A 12 cm. specimen taken from its natural habitat ate a large Corydalis larva; another newly captured 12 cm. specimen regurgitated a partly digested 6 cm. larva of its own kind.

During the first month after hatching, the vitelline veins of one side of the yolk sac degenerate, while those of the other side shift to a more nearly median position (fig. 197). Degeneration of the right or the left vitelline vein takes place in about an equal number of cases. It has already been noted that in those stages when the embryo lies continuously on one side the vitelline veins are best developed on the uppermost side; furthermore that the embryo falls on the right or the left side in about an equal number of cases. The facts strongly suggest that the position of the embryo during the period when the vitelline veins are developing is the factor that determines on which side the vitelline vein shall persist; but since making these observations I have had no opportunity to put the matter to a rigid test.

With the reduction of the yolk sac, the vitelline circulation suffers a corresponding diminution in extent; during the late stages of this process, through the increasing thickness and opacity of the ventral body wall the vitelline veins are somewhat obscured.

In the free-swimming larva, the heart beats more rapidly than was the case during embryonic development.

Within a week or two after hatching, the rapid growth of the front limb rudiments enables the larva to support itself in the normal position of the adult. One month after hatching, the front limbs have increased decidedly in length and possess the full number of digits (four). The form and position of the front limbs adapt them for use as paddles; by means of a simultaneous backward stroke they aid the larva in getting a quick start for swimming. The posterior limbs develop more slowly; at this time they are relatively short and as a rule possess but three digits, though in some cases the full number (five) are present. Ten weeks after hatching in all cases both pairs of limbs possess the full number of digits and are used in walking in the same manner as in the adult. The limbs are broad and flat, and in swimming at a moderate rate of speed are used as paddles. After the sixth month of larval development the posterior limbs surpass the anterior in size and strength. In the two-year-old specimens reared in the laboratory the limbs appeared weak and poorly developed as compared with newly captured specimens of the same age.

As in the adult, the tail is the principal organ of locomotion during rapid swimming. Up to five or six months after hatching the tail remains much larger in proportion to body-size than in the adult. In the year-old larva the tail is proportionally much smaller than in specimens ten weeks after hatching. In the two-year-old specimens reared in the laboratory the tail is smaller than in newly-captured specimens of the same age.

One month after hatching, pigmentation is greatly advanced and extends over the external gills; when viewed from above the larva is now nearly black. The ventral surface remains white and nearly transparent in the throat region, yellow in the region of the yolk sac. Six months after hatching, the dorsal surface shows large dark spots of unusually dense pigment, which are characteristic of all the later stages; the ventral surface is slightly pigmented. In the year-old larva, the dorsal surface is still nearly black, but with a few scattering inconspicuous yellow spots; the abdomen is grayish and the throat region almost white. In the two-year-old larva the general color effect is not so dark; the larva is taking on the variegated color pattern of the post-larval and adult stages.

In the month-old larva, shedding of the cuticle was observed for the first time. From this time on, the water of the aquarium becomes almost cloudy with detached portions of epidermis.

Soon after the hatching period, cilia disappear from the general surface of the body, but persist on the gills where a strong eddying current of water is produced. The gills remain ciliated until at least six months after the hatching period; at this point observations were necessarily discontinued.

As early as five months after hatching and frequently thereafter, larvae have been observed to come to the surface for air and to give up large bubbles of air from the mouth, indicating that the lungs are functional.

During the first year of larval life, the ventral portions of the three gill arches bearing the external gills are expanded into leaf-like plates, partly covered by the opercular lateral portions of the gular fold. At this time there are three gill openings. Toward the end of the first year the median portion of the gular fold disappears; but the lateral portion extends as a small opercular fold above the root of the anterior external gill. During the second summer the opercular fold extends dorsally far enough to cover the bases of all three external gills. With the loss of the external gills the opercular flap persists and partly roofs over a shallow cavity containing the leaf-like plates previously mentioned.

C. Post-larval stages

Sexual maturity is attained with a length of at least 30 cm. for the male, and 35 cm. for the female. During August, specimens 14 to 20 cm. in length have been very rarely taken, while specimens measuring from 20 cm. upwards are very plentiful; this suggests that the young ordinarily reach a length of at least 20 cm. at the end of the third year, and that they do not ordinarily become sexually mature until the end of the fourth year.

The immature post-larval stages resemble in coloration the young adults described in Part I. Aside from the loss of the external gills, the most conspicuous changes as compared with the larvae are a slight progressive dorso-ventral flattening of the body, and the gradual development of the folds of the skin which are so prominent in adult and especially in very large and presumably old specimens. A transverse slit-like spiracular opening is bordered anteriorly by the small opercular flap and posteriorly by a similar but still smaller fold of skin. The spiracle leads to a small cavity containing a single persistent gill opening bordered by two leaf-like plates; the other gill-lamella is greatly reduced or has disappeared.

D. Summary

The vitelline membrane is shed about the time of the closure of the neural folds.

Shortly after the closure of the neural folds, the entire surface of the embryo becomes ciliated. The beat of the cilia is in general toward the posterior end of the body; currents of water are produced which are subservient to respiration. After the appearance of the external gills, ciliary currents are especially strong in their immediate vicinity. Soon after the hatching period, the cilia disappear except on the external gills; here they persist until at least six months later.

Soon after the closure of the neural folds, the embryo falls on one side, where it lies until, in a much later stage, it is able to turn over through muscular activity. The embryo falls on the right or the left side in about an equal number of cases.

Rotation of the embryo, due to the beating of the cilia, commences before the embryo has fallen on its side but is more pronounced afterward. In most cases rotation proceeds in a clockwise direction when an embryo is lying on its right side, in an anti-clockwise direction when it is lying on its left side. Rotation may be of service in preventing adhesion of the embryo to the capsule.

The vitelline veins develop as paired structures along the sides of the yolk sac, and shift gradually toward the ventral median line. The vitelline veins develop more rapidly on that side of the yolk sac which happens to be uppermost. Soon after the hatching period, the vitelline veins of one side degenerate, while those of the other side reach their fullest development; the veins of the right or the left side persist in about an equal number of cases. Probably the position of the embryo during the stages when it lies continuously on one side is the factor that determines which set of vitelline veins shall gain the ascendancy.

The newly hatched larva retains a supply of yolk sufficient to last it from two to four months.

The tail of the early larva is proportionally much larger than in the adult.

Pulmonary respiration is established about five months after the hatching period.

The metamorphosis takes place at the end of the second year.

Sexual maturity is attained, probably at the end of the fourth year, with a body length of at least 30 cm. for the male and 35 cm. for the female.

X. TIME RECORD

For the characteristics of the different stages reference is made to the text and illustrations, especially the photographs. For methods used in obtaining the time record, see the introduction (Section VI). Table 1, page 534.

XI. ABNORMALITIES

The present section deals primarily with abnormalities found in embryos taken from their natural environment, or kept under conditions as nearly normal as possible.

1. *Large yolk plug*

In the early stages of development the most common abnormality is the presence of an unusually large yolk plug. Examples are shown in figures 198 and 199, though these are far from representing extreme cases. In well-marked examples the blastopore forms a complete circle only a little below the equator, and

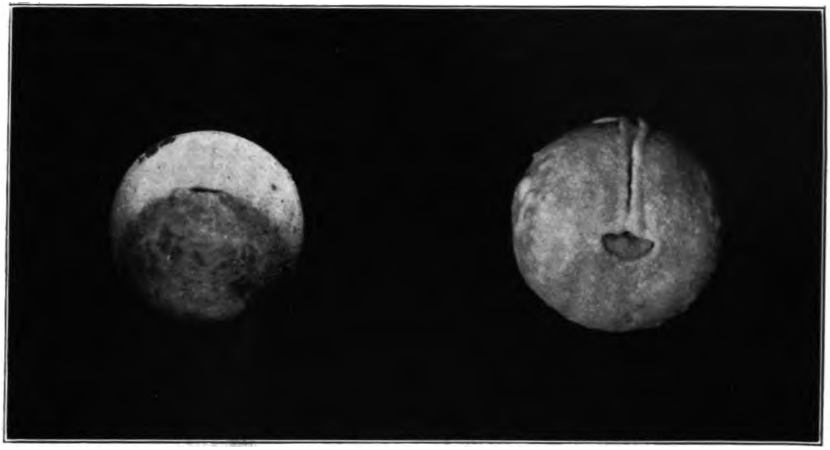
TABLE 1

STAGE	TIME AFTER FERTILIZATION	INTERVAL
1	24 hours (18 to 28)	
2	30 hours (24 to 36)	6 hours (5 to 7)
3	35 hours (29 to 44)	5 hours (4 to 6)
4	39 hours	4 hours (3 to 5)
5	43 hours	4 hours
6	47 hours	4 hours
7	51 hours	4 hours
8	63 hours	12 hours
9	3½ days	19 hours (14 to 24)
10	5 days	36 hours (24 to 48)
11	7 days (6 to 8)	48 hours
12	10 days	72 hours
13	11½ days	36 hours
14	12½ days	24 hours
15	13¼ days	18 hours
16	14 days	18 hours
17	15 days	24 hours
18	16 days	24 hours
19	17 days	24 hours
20	19 days	48 hours
21	23 days	4 days
22	4 weeks	5 days
23	6 weeks	2 weeks

Metamorphosis: Two years after fertilization.

a large yolk plug persists long after the closure of the neural folds; such embryos usually produce normal larvae, though spina bifida is an occasional result. In extreme cases the blastopore may form entirely above the equator; such embryos die before reaching the larval stage.

This abnormality appears most frequently in eggs that have been kept in jars of water during warm weather, and especially in material that has been shipped long distances. Probably it may be brought about by a variety of unfavorable conditions: heat, lack of oxygen, mechanical agitation, and injurious substances (e.g., cinders) in the water. It is readily produced by treatment with sodium chlorid; very marked cases of spina bifida may result.



198 199

Figs. 198 Postero-dorsal view of a gastrula of Cryptobranchus alleghenienis with an unusually large yolk plug. The blastopore extends around the egg as a complete circle, but only the dorsal portion shows distinctly in the photograph. From preserved material. × 4.

Fig. 199 Posterior view of an embryo shortly before the closure of the neural folds, showing persistent yolk plug. Photographed from preserved material. × 4.

2. Exovate abnormality

A rather common abnormality originating at the time of the formation of the neural groove may be called the exovate abnormality. A small protuberance on the site of the closed fenestra takes the form of a spherical exovate; in some cases this reaches a diameter about half as great as that of the egg. In most cases the extra-ovate remains connected with the egg by a very narrow stalk; the malformation is entirely extra-embryonic, the protruded yolk is gradually absorbed and the egg produces a normal larva. In other cases the protuberance takes the form of a dome-like swelling and increases in size until the egg collapses.

3. A double embryo

On September 27, 1906, I found a nest containing embryos in an advanced stage of development, and among them the double embryo shown in figure 200. The total bulk of this double embryo is about equal to that of an ordinary single embryo from the same spawning. That the occurence of such a monstrosity in this species is exceedingly rare in nature is shown by the fact that during the past seven years I have collected many thousands of embryos from nests, yet in no other instance have I found an abnormality even approaching the one under consideration.

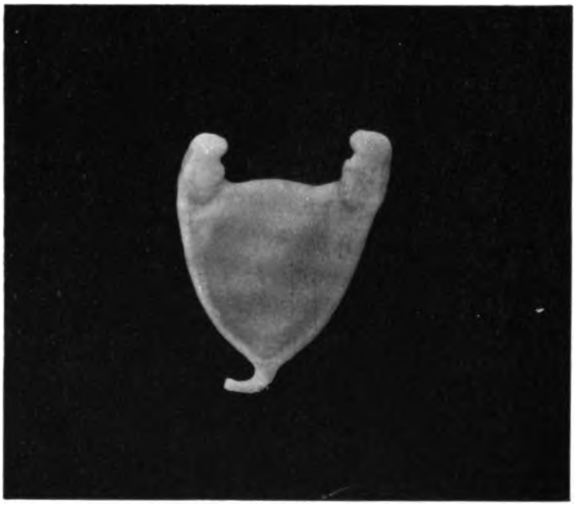


Fig. 200 Double embryo of Cryptobranchus allegheniensis. Photographed after preservation in formalin. × 4.

The precise manner in which the double embryo originated is problematical. Single capsules sometimes contain two eggs in close contact; but the small size of the double embryo precludes the possibility that it was formed by the union of two such eggs. Through treatment with sodium chloride I have produced embryos in which the brain differentiated without closure of the neural folds, but no true double embryos were obtained by this means; moreover in nature it is improbable that unusual chemical influences should affect a single egg.

It is more likely that the abnormality was brought about by mechanical means. A partial separation of the first two blastomeres (e.g., through the egg becoming lodged in a crevice) might in some cases result in the formation of a double embryo (Spemann '01 to '03). But owing perhaps to the heavily yolk-laden character of the egg, and the slowness with which the first furrow cuts its way through the yolk, my attempts to produce a double embryo in this manner have failed. When a fine silk thread or a hair was tied about the egg in the two-cell stage so as to constrict it in the plane of the first cleavage furrow, the egg usually burst before reaching the gastrula stage. In a single case the egg lived until after the formation of the neural folds, but developed a single embryo with its principal axis at right angles to the constricting cord.

A more probable explanation is that in the two-cell stage the egg became inverted and remained for some time in this position subject to the rearrangement of contents through the disturbing influence of gravity acting in a direction opposite to the normal (Schultze '95). But the whole question is complicated by the more fundamental problem of the determination of the median plane of the embryo. My experiments, to be described in a later paper, show that in Cryptobranchus the first cleavage furrow tends to form at right angles to the direction of entrance of the sperm (as in Triton, but not as in the frog where the first cleavage tends to coincide in direction with the path of the sperm). If in Cryptobranchus, as in the frog, the entrance path of the sperm lies approximately in the median plane of the embryo, then the conditions necessary for the production of a double embryo by the separation of the first two blastomeres must be of very exceptional occurrence.

4. *Spiral-tailed monsters*

In the fall of 1910, some embryos shipped by express to the University of Wisconsin and reared there in city water, acquired, in about 8 per cent of their number, the abnormality shown in figures 201 and 202. From the condition of the tail in late larval stages, specimens affected with this malformation may be designated as 'spiral-tailed monsters.' At first this peculiarity seemed merely the persistence of an embryonic condition; for in early stages the tail is always strongly flexed and in these particular cases it failed to straighten out. But as development progressed the tail became twisted into a pronounced spiral. The illustrations show the extreme condition; cases occur forming a series intermediate between this and the normal. In some cases the back is arched or humped. So far as can be judged from surface views, the entire abnormality seems to be brought about by the constricting effect of a band of tissue lying in the ventral median line.

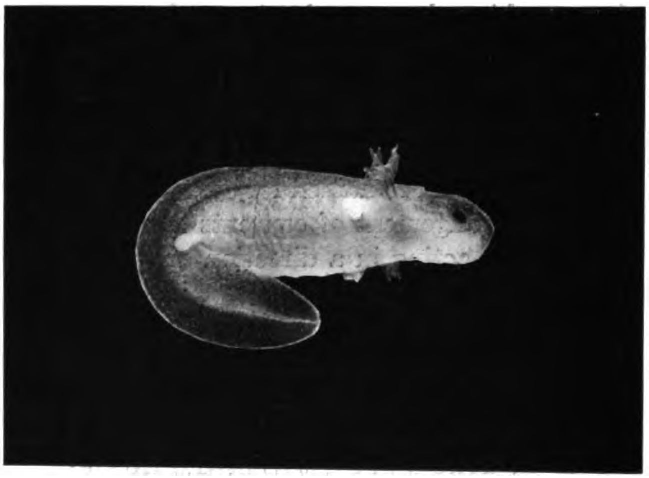


Fig. 201 Spiral-tailed monster of Cryptobranchus allegheniensis at the time of hatching. Photographed from the living embryo anaesthetized with chloretone. × 3.

As a result of this malformation, the larva is compelled to lie on one side, and can swim only in a grotesque fashion, with backward circling movements. Affected larvae take no food, and die shortly after using up their supply of yolk.

An analysis of the water was secured but the subject has not yet been further investigated. Stockard ('06, p. 119) noted the occurrence of a similar abnormality in Fundulus embryos.

5. *Critical periods in the life history*

In closing this section it remains to note that there are three stages in the life history characterized by unusual mortality—nature's examinations occur in these stages. These critical periods of development are:

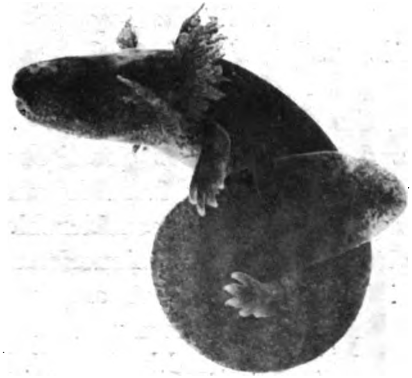


Fig. 202 Spiral-tailed monster of Cryptobranchus allegheniensis two months after hatching. Photographed from the living larva anaesthetized with chloretone. × 3.

(*a*) *The late blastula.* Many eggs seem unable to form a gastrula.

(*b*) *The hatching period.* Many embryos seem to lack the strength necessary to escape from the gelatinuous envelope, and die in the capsule.

(*c*) *The period of change in the method of nutrition.* After exhausting their supply of yolk, many larvae refuse to take food, or seem unable to set up digestive processes, and die of starvation.

XII. PHYLOGENY

We are here concerned with (a) the origin of the amphibia in general, (b) the origin of the urodeles, and (c) the interrelationships of the urodeles with special reference to Cryptobranchus. The evidence may be classified as (*a*) anatomical, (*b*) paleontological, and (*c*) embryological. In the following survey, no attempt is made to keep these lines of evidence strictly separate; for paleontology is simply an extension of comparative anatomy to fossil forms, with the added element of sequence in time; while the development during the late embryonic and larval stages gives the key to many adult structures that could not otherwise be homologized.

It is not within the scope of this paper to enter extensively into anatomical and paleontological questions; but as a check on possible generalizations derived from the study of amphibian embryology the writer has devoted considerable time to a study of the paleontological material in the American Museum of Natural History, and has endeavored to acquire some degree of familiarity with the results of modern research in this field. In this work he has been greatly aided by Dr. W. K. Gregory, who has in progress a detailed review of the origin of the amphibia (see abstracts in Science, Gregory '11 a, '11 b and '12).

The tetrapoda form a coherent group. The gap between this group and the fishes is one of the weak places in vertebrate phylogeny, and it is here that the idea of continuity in the descent of the higher vertebrates has been most often attacked; some have maintained a diphyletic origin for the tetrapods and fishes.

The amphibia undoubtedly include the most primitive known tetrapods. It is on this account that the amphibia possess a peculiar interest from a phylogenetic point of view; the problem of the origin of the amphibia is the problem of the origin of the tetrapods in general.

The fundamental unity of the gnathostome type leads us to look among the true fishes for the nearest living or fossil representatives of the ancestral stock of the amphibia. For in fishes and tetrapods the following features, as well as many others, are homologous: the chief divisions of the brain; the cranial nerves; the eye muscles and their innervation; the chondrocranium arising from trabeculae and parachordals, and including olfactory, optic and auditory capsules; the visceral arches; and the essential structures of the circulatory system. If we compare only the amphibia with the fishes the range of resemblances becomes still greater; all the important structures are in essential agreement except those concerned with the outer halves of the paired extremities.

The argument for a diphyletic origin of the pisces and amphibia is based upon (*a*) the lack of homology in certain elements of the skull, and (*b*) the difficulty that is experienced in deriving the amphibian limb from the fin of any known fish. But the resemblances in the skull bones become very close when we consider fossil forms, and the trend of increasing knowledge is in the direction of more complete homology rather than the reverse. Moreover we find difficulties almost as great in homologizing cranial elements in different fishes; for these structures are plastic and exposed to environmental influences, and with the radical change from an aquatic to a terrestrial habitat we should expect them to be profoundly influenced. The endoskeleton of the paired appendages presents us with a problem of greater difficulty but here, too, we have to deal with structures that we should expect to be greatly modified in connection with the change of habitat. In view of the wide range of resemblances in important structures one is hardly inclined to consider seriously the idea of a diphyletic origin for the fishes and amphibia.

The amphibians must have descended from some fish having scales with the potentiality of fusing into bony plates. The dermal bones forming the roof of the skull must have been arranged in pairs on each side of a median suture; for this is the condition found in the most primitive known amphibians (e.g., Branchiosaurus). The ancestral form must be sought in some fish having the endoskeleton of the paired fins widely protruded from the body, and with pectoral and pelvic members similar. Such fins functioned primitively as paddles; with the adoption of a terrestrial method of locomotion, by creeping or crawling on a more or less solid substratum, the endoskeletal elements of the limbs become greatly strengthened and progressively longer in order to lift the body from the ground. Such a progressive elongation of the limb bones, particularly the proximal elements, may be traced in both fossil fishes and amphibians; in the latter the pelvic girdle is also found becoming definitely articulated with the axial skeleton. The ancestral form must have been short-bodied; for in many groups of animals a progressive elongation of the body, culminating in eel-like forms, is found to accompany a degenerate structure scarcely capable of giving rise to higher forms (Gregory '07, Appendix I).

The lack of scales with the potentiality of fusing into bony plates is alone sufficient to exclude the elasmobranchs from the immediate ancestry. For affinities ancestral to the amphibia most authors have looked to the crossopterygii or the dipnoi. Both have dermal bones, and both fulfil the requirement regarding fins with widely protruded basal lobes and with endoskeletal elements from which the framework of true limbs might be derived.

At first sight the dipnoi seem best to bridge the gap between fishes and amphibia. For the lung-fishes have survived by virtue of an approach to the tetrapod type, enabling them to exist during periods of drought. But various considerations derived from the study of paleontology and comparative anatomy make it probable that these terrestrial adaptations were independently acquired, and that the dipnoi were already too highly specialized in other respects to give rise to the amphibia. The mosaic of small bones forming the greater part of the roof of the skull, particularly in the fossil representatives of this group (e.g., Dipterus), and the usual occurrence of one to several large median elements in this region, make it difficult or impossible to homologize the dermal bones of the skull with those of amphibia. The characteristic dentition is far removed from that of the amphibia. Marginal teeth, with exceptions in the cases of some very early forms (e.g., Phaneropleuron), are lacking; in the later forms the loss of maxillae, premaxillae and nasals shows a progressive tendency toward degeneration in these regions. The concentration of the teeth into tritoral clusters in the roof and floor of the mouth is not in itself an unfavorable feature, for vomerine teeth occur in the amphibia; but in all except certain very early fossil forms (e.g., Uronemus) the fusion of these teeth into large dental plates with grinding ridges has gone too far to give rise to the condition found in the amphibia. So far as the paleontological and anatomical evidence is concerned, the known facts tend to exclude the dipnoi from the direct ancestry amphibia, of the yet do not wholly preclude the possibility that future discoveries may supply us with more favorable material amongst early fossil forms. In so far as the terrestrial adaptations of the dipnoi resemble those of the amphibia, the case may be one of parallelism or convergence; their more fundamental resemblances indicate that they are not very far removed from a common ancestry.

Turning to the crossopterygii we find more favorable anatomical and paleontological grounds for comparison with the amphibia. The dermal elements forming the roof of the skull occur in paired series; a large number of cranial bones may be definitely homologized with those of the amphibia (see especially Baur '96; Moodie '08 a; for materials for further comparison see Goodrich '09, and Zittel '11). It is difficult to believe that identical relations in so many bones could have been independently evolved. With regard to the fins, we find examples of a bifurcated type of endoskeleton that makes a more favorable starting-point for a tetrapod limb than the archipterygial type of the dipnoi; e.g., see fig. 203 for the pectoral fin of Sauripterus, and Goodrich '09, p. 275, fig. 244, for the pelvic fin of Eusthenopteron; the pectoral fin of Eusthenopteron (Goodrich '09, p. 282, fig. 252) is not quite so favorable. But both of these forms belong to the Rhizodontidae, whose skull is not so favorable for comparison with the amphibia as the skull of some other crossopterygians; in no one form do we find all the conditions ideal for the derivation of the tetrapod type. The occurrence in Polypterus of two kinds of ribs, both the ventral or pleural ribs characteristic of the teleostomi and dipnoi, and the dorsal ribs characteristic of elasmobranchs and tetrapods, is a point emphasized by Baur

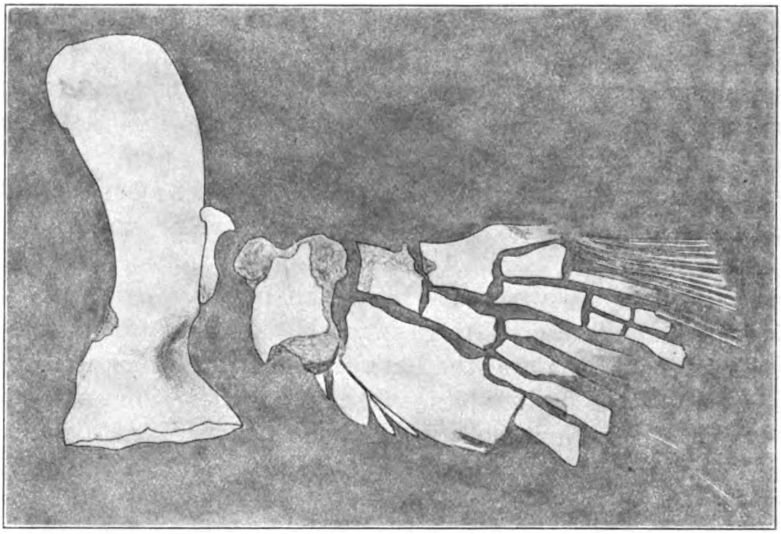


Fig. 203 Endoskeleton of the pectoral fin of Sauripterus taylori Hall, a crossopterygian from the upper Devonian. One-third natural size, linear reduction. From a drawing by Dr. L. Hussakoff of the American Museum of Natural History.

('96) as favoring a crossopterygian rather than a dipnoan ancestry for the amphibia.

It is upon embryological grounds that the strongest case has been made out for the derivation of the amphibia from the dipnoi; the known facts of development indicate the common origin and later separation of these two groups. In so far as this view is based upon a study of the early stages, the evidence may be dismissed with the remark that the early development of the crossopterygian Polypterus (Kerr '07 a) resembles that of the anura and the urodeles quite as much as does the early development of the dipnoi (Semon '00 and '01; Budgett '01; Kerr '00, '01 and '09); furthermore that these early stages are of very little value in connecting up the great groups of vertebrates. But some marked resemblances between dipnoi and amphibia in the later stages of development cannot be disregarded. Kellicott ('05, a and b), on the basis of a detailed study of the circulatory system of Ceratodus, came to the conclusion: "The resemblance in the vascular and respiratory systems between Ceratodus, the most primitive of the dipnoi, and the amphibia, especially the urodeles, are numerous and important, and cannot be explained as parallelisms." In this connection it is important to compare the development of the circulatory system of Polypterus. From the account given by Kerr ('07), it appears that in Polypterus the vascular system, particularly because of the presence of only a single pair of aortic arches, is decidedly less amphibian in character than that of Ceratodus.

In the present state of our knowledge it is impossible to reach an unqualified decision of the question under consideration. In weighing the evidence one should not forget that in numerous cases where anatomical and embryological evidence have come into conflict in deciding questions of the phylogenetic relationships of the larger groups of animals, it is the embryological evidence that has had to give way, and that recent anatomical evidence has to give place to paleontological. Whatever light may be shed by future discoveries on the question of the derivation of the amphibia from the crossopterygii or the dipnoi, it is clear that the point of origin is not far from either stock; in other words, that the three lines of descent have separated from a common stem at no very great intervals.

Concerning the immediate ancestry of the living amphibia we have detailed evidence only in the case of one group, which fortunately for our purpose is the caudata or urodela. According to Moodie ('08 b) the urodeles are descended from the branchiosauria, a group of primitive extinct amphibia from the carboniferous and Permian. These are small, short-tailed amphibians with broad heads. The skull is slightly more complex than in the urodeles, and there is a dermal exoskeleton consisting of rows of thin semi-cycloid scales, especially on the flanks and under side of the body. External gills are present in the larvae. The view that these forms are ancestral to the urodeles is based on a detailed comparison of the structure of the skull, the structure and form of the vertebrae and the ribs, the number of digits, the arrangement of the phalangeal elements, the character of the pectoral and pelvic girdles, the distribution of the lateral line system, the structure and form of the long bones, and finally the shape of the body. According to Moodie the caudata are degenerate branchiosaurians and the changes which have taken place in the exoskeleton are mostly brought about by the loss of certain parts. The urodele skull is especially degenerate in the occipital and temporal regions; it may be derived from the skull of Branchiosaurus (for which see Credner '81 to '90) by the loss of the dermoccipitals (supraoccipitals of Moodie '08; postparietals of Zittel '11), supratemporals (Zittel '11), postfrontals, postorbitals, sclerotics, epiotics (tabulares of Zittel '11), jugals and quadratojugals. The urodele skull has also in many cases become narrow in connection with a general elongation of the body—a degenerate feature. Hand in hand with the loss of dermal elements in the skull has gone the loss of the exoskeleton of the body.

The paleontological history of the apoda is unknown; but existing members of the group are in certain respects more primitive than the urodeles and more nearly allied to the stegocephali. Thus the hyoid and branchial apparatus is more primitive than that of any other recent amphibia; dermal scales are present which are probably homologous with those of the stegocephali. As in the urodeles, the skull shows degeneration in the loss of certain bones; but the epiotics (tabulares) are retained, and occasionally the postfrontals and the lacrimals. A second row of teeth is sometimes present on the mandibles. Aside from some very degenerate features, in other respects the apoda are highly specialized, indicating, as in the case of the anura, a line of descent separate from the urodeles. Yet there are some very suggestive resemblances. Attention has already been directed (Smith '12) to the marked similarity in structure of the egg envelopes of Ichthyophis to those of Cryptobranchus and Amphiuma, and to the likeness in the brooding habits; but in Ichthyophis the female protects the eggs, in Cryptobranchus the male. In their embryological development the apoda show many points of similarity to the reptiles.

With this background, we now come to the question of the interrelationships of the urodeles. In particular we are concerned with the phylogenetic position of the aquatic urodeles (the perennibranchs and the derotremes[2]) as related to the land-living salamanders.

The most prevalent view has been that the aquatic urodeles are the most primitive. Parker and Haswell ('97, vol. 2, p. 291) have said: "The perennibranchiate urodeles are undoubtedly the lowest of existing amphibia; they lead up, through such forms as Amphiuma, with persistent gill slits but deciduous gills, to the land salamanders, in which a purely terrestrial form is assumed." In a group standing between fishes and the typically terrestrial vertebrates, it is natural to regard the aquatic forms as transitional to the terrestrial. In fishes there are usually five branchial arches, and the gill slits remain open throughout life. The land-living salamanders have in the adult state only two branchial arches (Parker '77; Wiedersheim '77; Cope '89), and the gill slits are open only to the end of larval life. In the aquatic urodeles there are usually three or four branchial arches (as in the larvae of the terrestrial forms), and the gill slits usually remain open throughout life—conditions intermediate between fishes and salamanders. In view of the occurrence of external gills in all larval forms, the persistence of such gills in the perennibranchs might, on the recapitulation theory, be regarded as a primitive character.

In the opinion of various authors, the above interpretation represents a short-sighted view of the matter. Boas ('81) was the first to assert that the perennibranchs are larvae that have lost the ability to transform; this conclusion was reached as a result of a comparative study of the circulation. Cope ('85) described the retrograde metamorphosis of Siren, and concluded that the present Sirens are descendants of a terrestrial type. Gadow ('01, p. 66) suggested a terrestrial ancestry for the urodeles. Kingsley ('01) says: "The salamandrina form the central urodele stem, and the perennibranchs and derotremes have been derived from this stem by degeneration and the retention of larval characters." Kingsbury ('05), on the basis of a comparative study of the cranial elements, came to the tentative conclusion that Necturus is a permanent larva. The generalization formulated by Boas has recently been reiterated and expanded by Versluys ('09); briefly stated, his views are as follows:

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[2] The classification of Stannius ('56) is here followed, as it seems best adapted for the purposes of this discussion.

The great resemblance of the perennibranchs to salamander larvae is only a consequence of the fact that the first are also larvae, but larvae which no longer come to full development as their ancestors did; the metamorphosis is imperfectly undergone or wholly omitted. Nevertheless these larvae become sexually mature, as in neoteny. In the course of time, in adaptation to their aquatic habitat, they have become degenerate in many respects. The derotremes are salamanders that have become fixed in the transitional stage or metamorphosis. Thus the aquatic urodeles have a terrestrial ancestry; they are forms which have reverted to an aquatic mode of life.

The probable course of events giving rise to the perennibranchs may be described as follows: While the mature salamanders are constructed after the fashion of a land animal, their larvae live in water and in the course of time have become more and more adapted to aquatic life. They have extended their larval organization and thus increased the difference, which must be overcome by metamorphosis, between the larva and the grown-up animal. In time some organs show arrested development or degeneration of such a sort that the larvae can no longer develop into land-living salamanders; they remain life-long water dwellers. The condition is one of fixed neoteny (Boas '96). According to this view the perennibranchs are disconnected from one another and have evolved as neotenic larvae from different salamanders.

Confirmatory evidence for this view comes from the studies of Emerson ('05) on Typhlomolge. In some details of its structure this animal shows a remarkable similarity to the larvae of Spelerpes ruber. Probably Typhlomolge has been derived from the neotenic larva of some salamander of the family (Plethodontidae) to which Spelerpes belongs, adaptations to a subterranean water life having been added.

The derotremes also are not primitive urodeles. Since they show a mixture of the characters of the larvae and grown-up salamanders, one cannot derive them from typical larvae. Presumably the derotremes are descended from typical salamanders which have returned completely to aquatic life. Thus the metamorphosis, which serves to adapt the larvae to land life, has lost its biological meaning; it extends over a longer time, and finally sexual maturity overtakes the yet imperfectly built animal. Some organs complete the metamorphosis, others retain wholly or in part the larval condition. So in its skull Cryptobranchus (Reese '06) resembles the grown-up salamander; in its circulation it retains larval characters. Versluys believes Cryptobranchus to be descended from the amblystomidae.

If we take into account only living forms, much of the data thus far considered in seeking a solution of this general question of the relationship of the aquatic and land-living urodeles may be read either way. The key to the situation lies in the comparison of the structures of existing urodeles with those of fossil forms. We have seen that the extinct ancestor of the urodeles was probably an animal whose skull was more complete than that of any living urodele. In Necturus (Kingsbury '05), there is a considerable reduction in the number of cranial bones, comparable to the condition in the larval Spelerpes, and contrasting with the condition in the adult Spelerpes. If the stem-form of the urodeles was an animal like Branchiosaurus, then so far as the cranial elements are concerned the salamander, and not the perennibranch, is the more primitive form. There are five branchial arches in Branchiosaurus—a condition which does not exclude either view of the interrelationships of the urodeles. Branchiosaurus had external gills only in the larval stage; this indicates that the condition found in the perennibranchs is probably not in the ancestral line of the caducibranchs (salamandrina).

Von Eggeling ('11), from a comparative study of the histogenesis of the skeleton of the limbs of urodeles, favors the view that the perennibranchs, derotremes and siredon are derived from the caducibranchs.

With the transition from the water to the land, or vice versa, one might expect important modifications in the sense organs. In the amphibia this expectation is best realized in the auditory organs, which in the form of a columellar apparatus fitting into a fenestra ovalis here occur for the first time in the vertebrate series. From a study of the modifications of this apparatus we may well hope to obtain information concerning the more intimate phylogenetic relationships within the group. According to the recent work of Kingsbury and Reed ('09) on the urodeles, there is close correlation between the type of auditory apparatus present and the habits (aquatic, semi-aquatic, terrestrial, burrowing, etc.) of the animals. From their results one may note many resemblances between the adults of the typically aquatic forms and the aquatic larvae of the terrestrial forms, as well as contrasts between both of these and the adult terrestrial forms. Concerning the phylogenetic relationships as indicated by the auditory apparatus Reed ('09) says:

> Cryptobranchus is the most generalized. The amblystomidae are intermediate between Cryptobranchus and all other groups. The plethodontidae and desmognathidae are departures from the Amblystoma stem while from these the sirenidae and Amphiuma seem to be degenerated. Diemyctylus and Triton are identical with regard to these ear structures and differ from all others. They are to be considered the most specialized. Between Diemyctylus and Triton on the one hand and the amblystomidae on the other Salamandra stands intermediate, resembling more strongly the amblystomidae.

If the generalized condition is really a primary one, then so far as the evidence from this single character is concerned, Cryptobranchus is one of the most primitive of urodeles; the evidence is not in line with the hypothesis of Versluys. But one should seriously consider whether the correlation between the sound-transmitting organs and the environmental relations is not too close to make the character of much phylogenetic value.

Osborn ('88) states that in Cryptobranchus we have the most primitive type of brain thus far observed among the amphibia. But it is not clear that the simple condition found is necessarily primitive.

Whipple ('06), from a comparative study of the ypsiloid apparatus in urodeles reaches the following conclusions:

(a) "That forms with lungs but without vestiges of an ypsiloid apparatus, and with no evidence of degeneration in the pelvic region (e.g., Necturus) are neither degenerate forms, nor permanent larvae of any of the salamandrina."

(b) "That the presence of a functional ypsiloid apparatus in Cryptobranchus indicates that Cryptobranchus lies near the line of descent of the salamandrina."

In summing up the facts for and against the hypothesis of the phylogenetic relationships of the perennibranchs, derotremes and salamandrina as outlined by Versluys, it seems to me that the arguments in favor of the hypothesis are founded on characters of greater phylogenetic value. In the reptiles and mammals, land forms are always primitive, aquatic forms secondary (Osborn '02). To the writer the evidence seems convincing in favor of a similar view for the recent urodeles. It might be added that pentadactylous limbs, more or less perfectly developed in fossil as well as recent amphibia, were undoubtedly produced in connection with terrestrial habits; and it should be emphasized that the forms ancestral to the present-day aquatic urodeles were probably not purely terrestrial, but passed through an aquatic larval stage, as in Branchiosaurus and most of the living salamanders.

It remains to consider briefly the phylogenetic value of some of the facts concerning the life cycle of Cryptobranchus that are embodied in the present contribution, and to discuss their bearing on the subjects just treated from a historical point of view. In an investigation thus far confined mainly to the external features of development, manifestly little more than a beginning can be made in such an interpretation.

The repeated failure of embryological generalizations to solve some of the larger phylogenetic problems led to a widespread reaction against the earlier too sweeping conclusions based on the recapitulation theory. The reproductive processes, while so fundamental, are very plastic, modified in closely related species and even changing somewhat in the same species kept under different conditions. A strict application of the biogenetic law would imply that the early stages of development are exclusively palingenetic; but from the earliest stages we find adaptations (e.g., the presence of yolk) that are prospective in their significance and have to do with distinctively larval phases, while the larval characters may be highly coenogenetic. It is the failure to distinguish between palingenetic and coenogenetic features of development that is mainly responsible for bringing the recapitulation theory into disrepute. With a clearer recognition of the limitations in this field of study, we may yet question whether the reaction against the validity of the recapitulation theory has not gone too far. As compared with anatomy and paleontology, embryology is doubtless of little service in connecting up the great groups of animals; yet it is indispensable in the solution of many special problems.

In a comparative study of the breeding habits (Part I, Smith '12) we must remember that we are dealing with characters that occur very late in the ontogeny, having to do with the adult rather than with the developing animal. Consequently such characters are of no more phylogenetic value than the habits in general and the family, generic and specific morphological characteristics of the adult; they are exceedingly plastic and of value for comparison only within a very limited range of forms.

A phylogenetic interpretation of the methods of fertilization in urodeles has been given in a previous paper (Smith '07 b); it is here referred to with the remark that in view of the conclusions reached in the present paper regarding the trend of evolution in the urodeles, the series should be reversed. If we could go back far enough in the phylogeny of the vertebrates, doubtless we should find external fertilization to be the primitive condition (e.g., as in Amphioxus); but it is entirely possible that in Cryptobranchus the method is secondarily acquired.

As we should expect, the brooding habit of Cryptobranchus is very similar to that of the closely-related Amphiuma. The absence of a brooding habit in Necturus is noteworthy. Brooding habits very similar to that of Cryptobranchus are found in Desmognathus and Plethodon, but with this important difference: in these latter forms the female, not the male, cares for the eggs.

The egg capsules of the urodeles, as in other groups, show generic and even specific differences (e.g., the specific differences in the egg masses of Amblystoma, Smith '11 b). This is what might be expected, since the capsules are the product of the soma, not of the germ cells; the facts are in no way incompatible with von Baer's law. The close resemblance between the egg envelopes of Cryptobranchus and Amphiuma accords with the systematic relationship. Amongst terrestrial urodeles there is no close approach to the type found in Cryptobranchus; but in general we should seek for affinities in forms having the egg capsules more or less independent in the cluster, connected by stalks (e.g., as in Desmognathus, Wilder '99), rather than in forms in which the individual capsules are surrounded by a common jelly mass, as in Amblystoma.

The origin of the follicle cells of Cryptobranchus has been traced (Part I) from the epithelial cells of the ovarian wall. Hence these cells belong to the soma, and the rather marked differences between the follicle cells of Cryptobranchus and Necturus are of value for comparison only with a very limited range of forms.

Taking up the history of the egg proper, we first note that the progressive change of the ovarian egg from an alecithal through an isolecithal to a telolecithal stage is a recapitulation of a very ancient series of events in the phylogeny. The actual amount of yolk present is a coenogenetic character, for the yolk content changes greatly within nearly related forms.

The factors that determine the amount of yolk present in the eggs of different species are complex and do not readily fall under any single law. Protection of the eggs through nesting and brooding habits makes possible a reduction in their number, enabling the female to endow each egg with a larger store of yolk, thereby giving the young a better start in life. Such a store of yolk allows development to go further before the young animal is cast upon its own resources, so that the necessity for peculiarly larval adaptations is minimized. In purely terrestrial non-placental forms a large amount of yolk is certainly necessary, for the food (e.g., insect larvae) of the young is larger and less easy to capture than is the case with aquatic larvae. On the other hand a large store of yolk is common in the eggs of fishes. The most we can infer is that the presence of a large amount of yolk in the egg is one of the conditions that makes possible the invasion of the land; it cannot be said that the presence of an unusual amount of yolk in the egg of Cryptobranchus is evidence either for or against a terrestrial ancestry.

Among the known eggs of urodeles that of Cryptobranchus is probably the most heavily yolk-laden; the egg of Necturus contains nearly as much yolk. In the salamandrina one notes the large amount of yolk in the eggs of Desmognathus (Wilder '04; Hilton '04 and '09); Spelerpes (Goodale '11) and Plethodon (Piersol '09).

The absence of pigment in the egg of Cryptobranchus is correlated with the nesting habits, whereby the eggs are protected from the light. Similar conditions are found in Necturus, Desmognathus, Plethodon and Spelerpes.

The occurrence of a protoplasmic mantle and cytodisc in the late ovarian egg of Cryptobranchus parallels a condition in the teleost egg which reaches its full expression just before fertilization; in Cryptobranchus this condition is transient. The segregation of a definite layer of cytoplasm close to the surface of the blastodisc in Cryptobranchus shortly after fertilization suggests a parallel with the marked increase in thickness of the germinal disc of the teleost egg immediately after fertilization; Professor Dean informs me that he has observed a similar phenomenon in ganoids.

The fundamental features of the early embryonic development have to do with the building up of the very general structures and body relations common to all vertebrates. In their most general aspects cleavage and gastrulation are extremely palingenetic phases of development. But we find secondarily imposed on the essential features of these processes, modifications which are highly coenogenetic and of adaptive significance mainly for the embryo and larva (Lillie '98), rather than for the adult.

These stages seem to possess few characters intermediate between the extremes indicated, consequently they tell us little about the relationships of the larger groups; but the coenogenetic characters, which are plastic and vary widely within a limited range, may be of value for determining relationships within these groups. The most striking of these coenogenetic characters are correlated with the presence of yolk (Conklin '07).

In the highly telolecithal and heavily yolk-laden egg of Cryptobranchus, we may interpret holoblastic cleavage as a persistent primitive character. For were the ancestral form one with meroblastic cleavage, we should hardly expect the holoblastic method to arise under such unfavorable conditions.

Comparisons between the early cleavage furrows of different urodeles seem justified on the ground that we are comparing cells of the same generation; but in view of the highly indeterminate character of the cleavage (Jordan and Eycleshymer '94) such comparisons do not take us very far. Mechanical factors doubtless play a part (McMurrich '94), but these mechanical factors are conditioned by the organization of the egg, which is hereditary.

There is close correlation between the method of third cleavage and the yolk content. A vertical third cleavage is characteristic of heavily yolk-laden and highly telolecithal eggs; a latitudinal third cleavage is found rather in eggs with yolk both smaller in amount and with a lesser degree of segregation from the cytoplasm. Morgan ('93) has shown that in teleost eggs from which yolk has been experimentally removed, the third cleavage often comes in latitudinally, yet the eggs produce perfect embryos. Marked variation in the direction of the third cleavage furrows occurs in eggs in which the conditions are intermediate in character; the egg is oscillating between two possible modes of cleavage. A vertical third cleavage is characteristic of the egg of Cryptobranchus; it is found less uniformly in the eggs of Desmognathus; in Necturus and Diemyctylus the third cleavage is irregular; in Amblystoma it is latitudinal. So far as this character is concerned Cryptobranchus lies nearest to Desmognathus and is most remote from Amblystoma.

The important features of the later stages of the cleavage have to do with processes that are not well expressed in the superficial cleavage pattern: migration of cells and the various processes of differentiation that lead up to gastrulation and early embryo-formation.

The occurrence of a septal furrow in the gastrula stages of two such widely-separated forms as Cryptobranchus and Petromyzon is a remarkable case of convergence in purely embryonic characters. The septal furrow and fenestra are the mechanical product of gastrulation by invagination and epiboly in a heavily yolk-laden egg with a very thin roof to the blastocoële. So far as known these features of gastrulation in Cryptobranchus are unique among urodeles; but there is evidently an approach to this condition in Spelerpes, since the egg is heavily yolk-laden and during gastrulation the blastocoele roof becomes quite thin (Goodale '11).

The study of the later embryonic and larval stages is as yet too superficial to furnish much data for phylogenetic generalization; yet for this purpose the late stages will probably prove of greater value than the earlier ones (Wilson '98, p. 23). It should be particularly noted that the larval Cryptobranchus reaches an age of two years before transforming—evidence of a retarded metamorphososis. Reasons have already been given for believing that the metamorphosis is incomplete.

The study of the breeding habits, the organization of the eggs, and the early course of development lead us to look among the land-living salamanders for affinities to Cryptobranchus—more particularly to forms like Desmognathus, Spelerpes and Plethodon. Considerable evidence from comparative anatomy, particularly with regard to skull structure, will be found to harmonize with this view. Desmognathus in particular is suggestive; according to Kingsbury ('02) it is semi-aquatic in its habits, living at the edges of swiftly running brooks. It conceals itself under stones at the edge of the stream or in its immediate vicinity, and here its unpigmented and heavily yolk-laden eggs are laid. There is a brooding habit, though in this case the female guards the eggs; we have noted some marked similarities to Cryptobranchus in the early development. Yet we are hardly warranted in concluding that the relationship is very close. In particular we should expect the immediate ancestral stock of Cryptobranchus and the closely related miocene fossil Andrias scheuchzeri (Gadow '01, p. 84) to consist of larger animals.

According to the view adopted in this paper, the urodeles, very remotely descended from aquatic stock, are primarily terrestrial, but with aquatic larvae. On the land they were unsuccessful in the struggle for existence in the open; they took refuge in sheltered situations, and for this they have paid the penalty of degeneration. Yet, in the main, the result of the arrest of a typical terrestrial adaptive radiation has been the retention of primitive characters. Some became secondarily aquatic; this is one phase of the tendency toward secluded habits, and involves the retention of larval characters. Added to these more conspicuous peculiarities we find a great variety of special adaptations to a retired mode of life, some of them correlated with the defenseless condition of the animals. Of all these changes, reversion to an aquatic mode of life is a factor which, cutting across many lines of descent, has done most to disguise the real relationships, and of this we have a conspicuous example in Cryptobranchus.

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PLATES

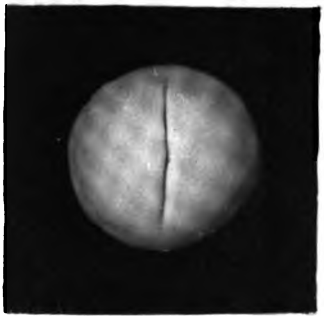
PLATE 3

EXPLANATION OF FIGURES

(Cryptobranchus allegheniensis)

All the figures are from preserved material, fixed in bichromate-acetic-formalin excepting the eggs shown in figures 210 and 212 which were fixed in formalin. The animal hemisphere is shown in every case. × 4.

204 Stage 1. First cleavage.
205 Stage 2. Second cleavage.
206 Stage 3. Third cleavage.
207 Stage 4. Fourth cleavage.
208 Stage 4. Fourth cleavage. Figure 82 is drawn from the same egg.
209 Stage 4. Fourth cleavage. Figure 81 is drawn from the same egg.
210 Stage 5. Thirteen micromeres. Figure 87 is drawn from the same egg.
211 Stage 5. Seventeen micromeres. Figure 86 is drawn from the same egg.
212 Stage 6. About thirty-two micromeres. Figure 98 is drawn from the same egg.
213 Stage 6. About thirty-two micromeres.
214 Stage 6. About thirty-six micromeres.
215 Stage 7. About sixty-four micromeres.



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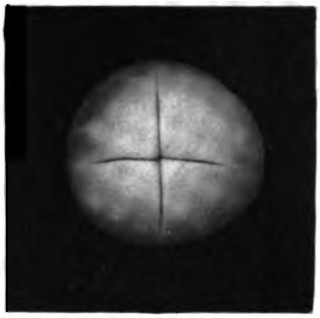

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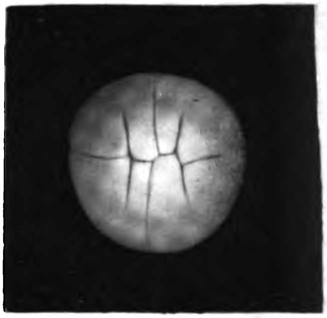

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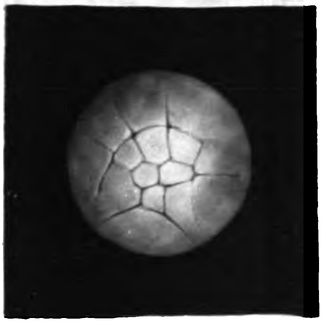

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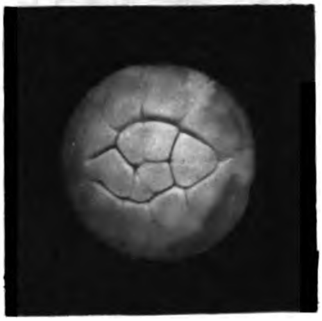

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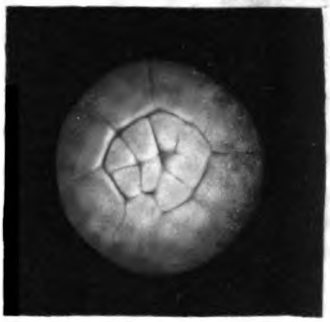

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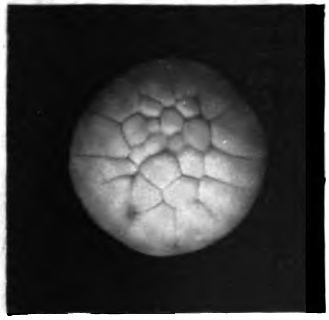

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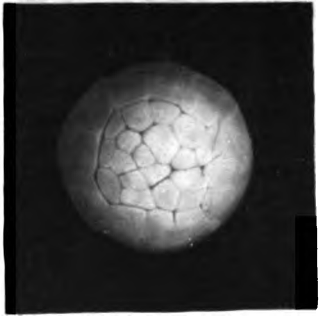

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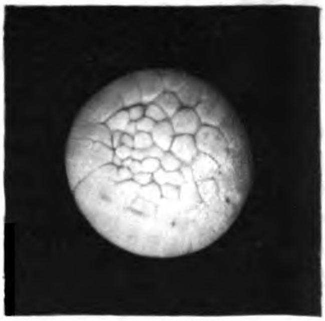

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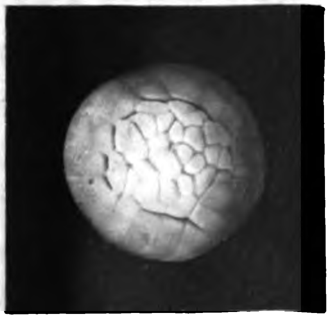

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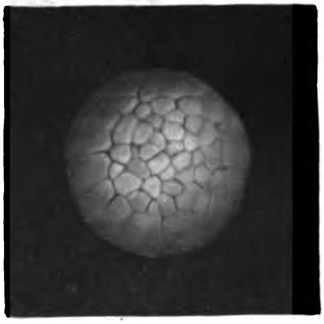

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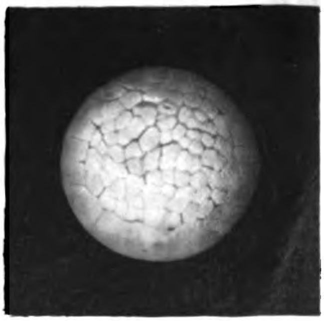

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PLATE 4

EXPLANATION OF FIGURES

(Cryptobranchus allegheniensis)

All the figures are from preserved material fixed in bichromate-acetic-formalin. × 4.

216 Stage 7.5. Equatorial view.

217 Stage 8.0. Upper hemisphere.

218 Stage 9.5. Equatorial view.

219 Stage 10.5. Equatorial view of an embryo nearly ready for gastrulation.

220 Stage 11.4. Early gastrula, showing the dorsal lip of the blastopore.

221 Stage 11.5. Antero-dorsal view of a gastrula, showing the fenestra.

222 Stage 11.6. Anterior view of a gastrula, showing the fenestra.

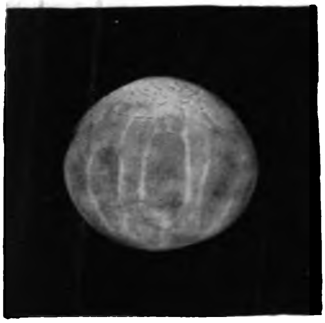
223 Stage 12.4. Dorsal view showing early neural plate and neural groove.

224 Stage 12.4. Posterior view of the same embryo as in the preceding figure, showing the yolk plug and a part of the neural plate and neural groove.

225 Stage 12.5. Dorsal view showing the neural groove and the dorsal lip of the blastopore. Figure 143 is drawn from the same embryo.

226 Stage 13.4. Dorsal view showing early neural folds.

227 Stage 13.5. Posterior view showing the form of the late blastopore, and the posterior part of the neural plate.



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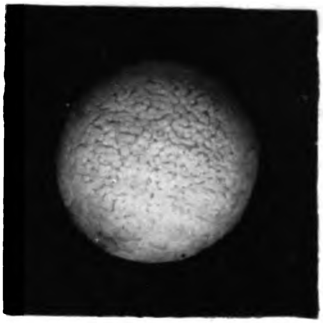

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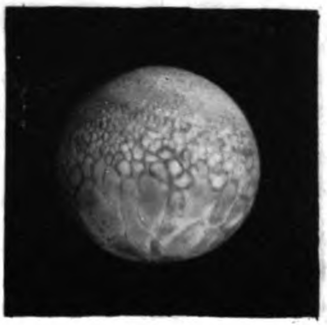

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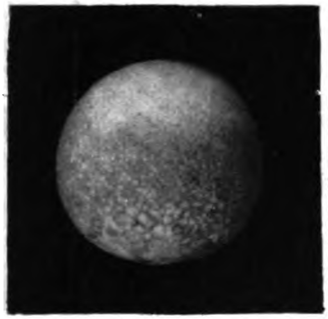

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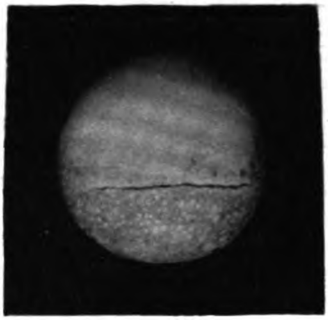

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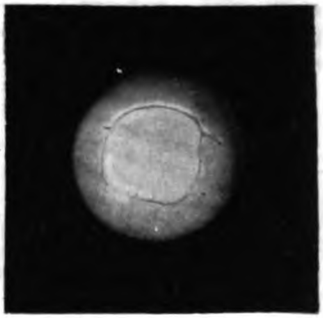

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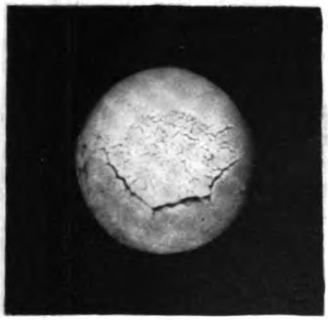

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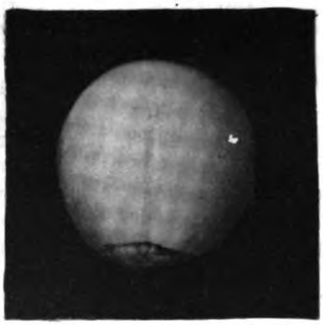

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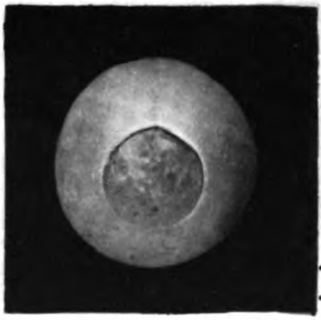

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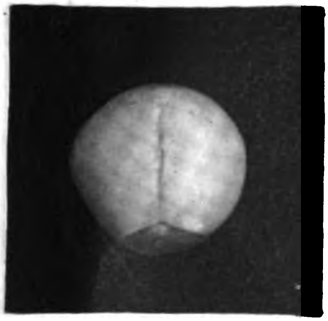

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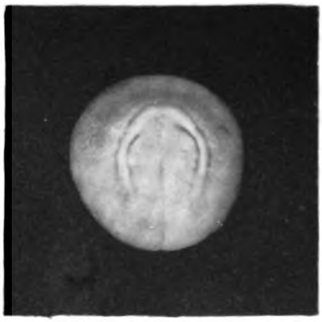

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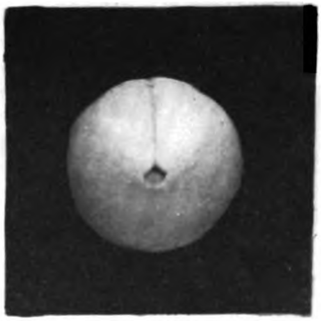

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PLATE 5

EXPLANATION OF FIGURES

(Cryptobranchus allegheniensis)

Bichromate-acetic-formalin fixation. × 4.

228 Stage 13.5. Posterior view showing the form of the late blastopore, and the posterior part of the neural plate.

229 Stage 14.1. Dorsal view showing neural folds and the early segmentation of the neural plate. Figure 166 is drawn from the same embryo.

230 Stage 14.1. Postero-dorsal view of the same embryo as in the preceding figure, showing the late blastopore and the segmentation of the neural plate.

231 Stage 14.9. Posterior view, showing the late blastopore. Figure 176 is drawn from the same egg.

232 Stage 14.9. Dorsal view of the same embryo as in the preceding figure, showing the segmentation of the neural plate.

233 Stage 15.4. Dorsal view. Figure 184 is drawn from the same egg.

234 Stage 15.8. Dorsal view. Figure 182 is drawn from the same egg.

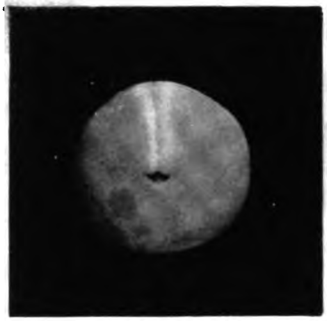
235 Stage 16.0. Dorsal view showing closing neural folds.

236 Stage 16.8. Dorsal view.

237 Stage 16.8. Antero-dorso-lateral view of the same embryo as in the preceding figure.

238 Stage 17. Antero-dorsal view (inverted with respect to the natural position).

239 Stage 17. Lateral view.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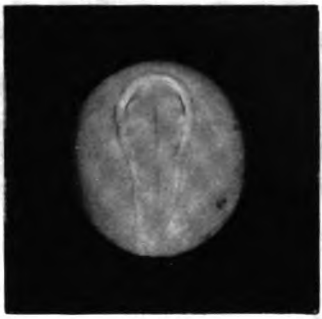
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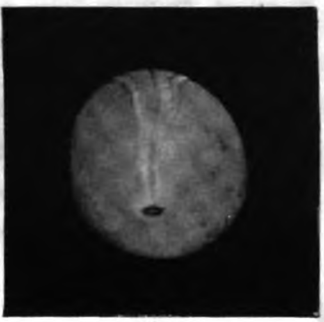
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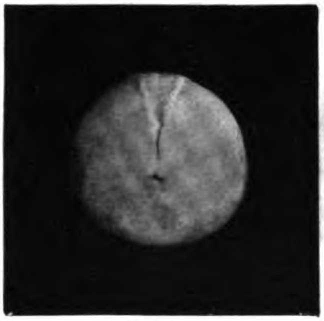
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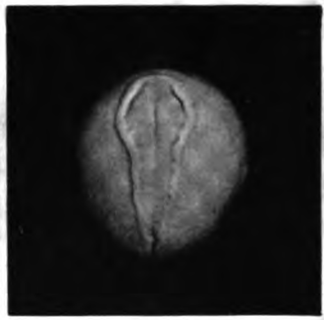
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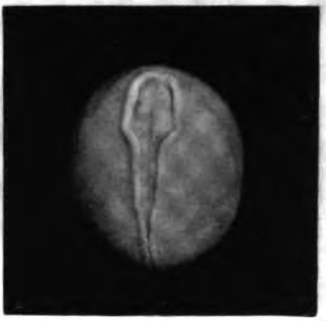
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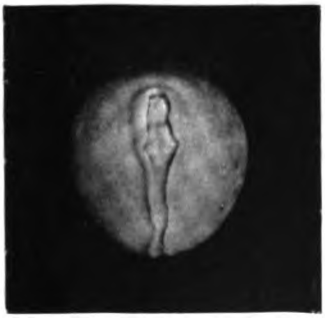
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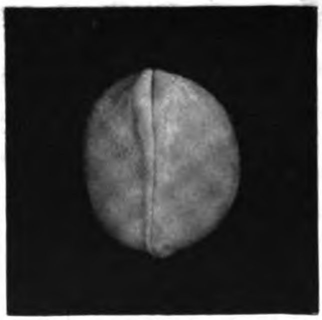
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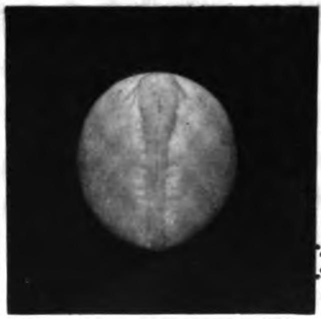
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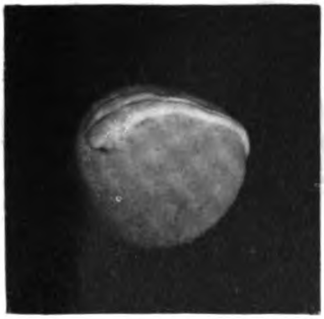
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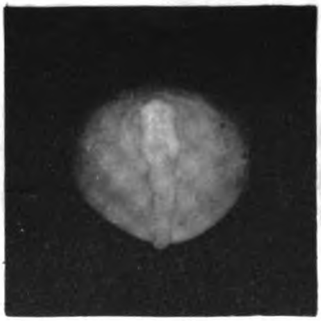
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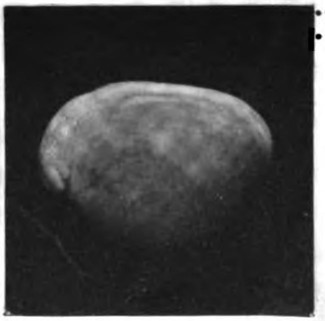
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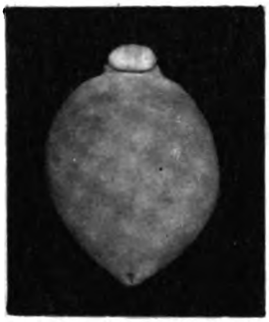
PLATE 6

EXPLANATION OF FIGURES

(Cryptobranchus alleghenienis)

Bichromate-acetic-formalin fixation. × 4.

240 Stage 17. Ventral view of the embryo shown in the preceding figure.
241 Stage 17. Dorso-lateral view of the embryo shown in the preceding figure.
242 Stage 17. Dorsal view of the embryo shown in the preceding figure.
243 Stage 18. Ventral view.
244 Stage 18. Lateral view of the embryo shown in the preceding figure.
245 Stage 19. Lateral view.
246 Stage 19. Dorsal view.
247 Stage 19. Lateral view of the embryo shown in the preceding figure.
248 Stage 19. Lateral view.
249 Stage 20. Lateral view.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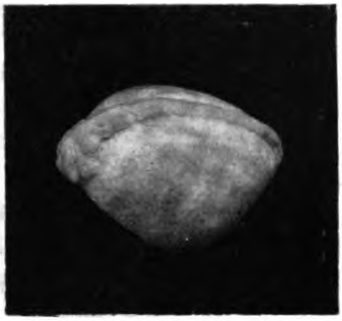
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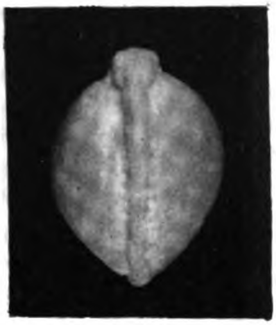
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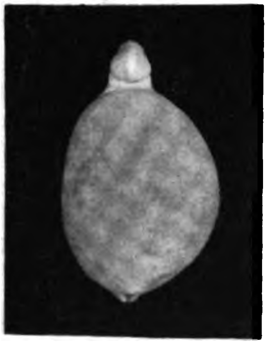
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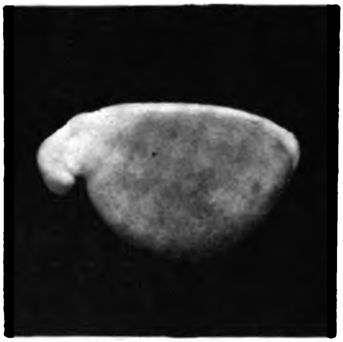
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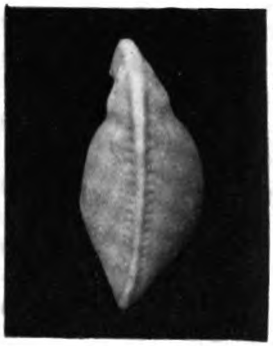
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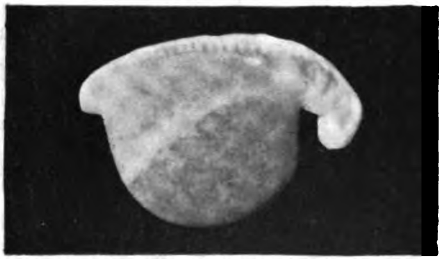
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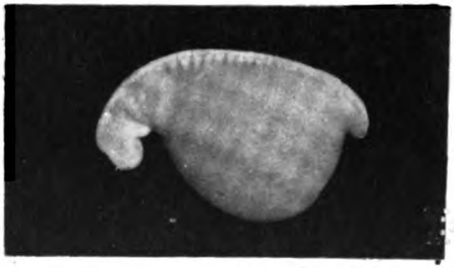
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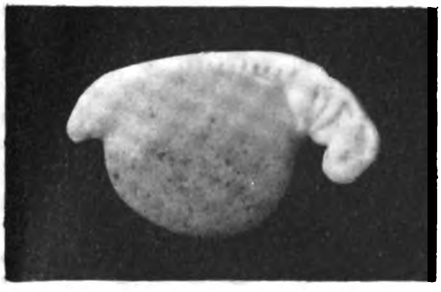
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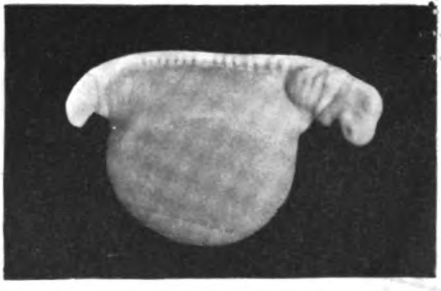
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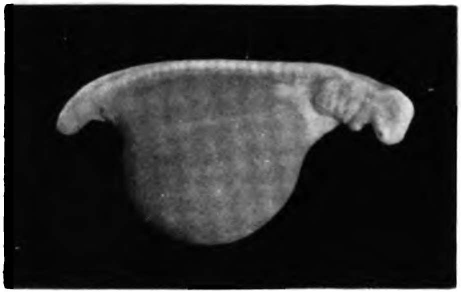
PLATE 7

EXPLANATION OF FIGURES

(Cryptobranchus alleghaniensis)

Bichromate-acetic-formalin fixation. × 4.

250 Stage 20. Lateral view.
251 Stage 20. Ventral view of the embryo shown in the preceding figure.
252 Stage 21. Ventral view.
253 Stage 21. Lateral view.
254 Stage 21. Dorsal view of the embryo shown in the preceding figure.
255 Stage 21. Ventral view of the embryo shown in the preceding figure.
256 Stage 22. Dorsal view.
257 Stage 22. Ventral view of the embryo shown in the preceding figure.



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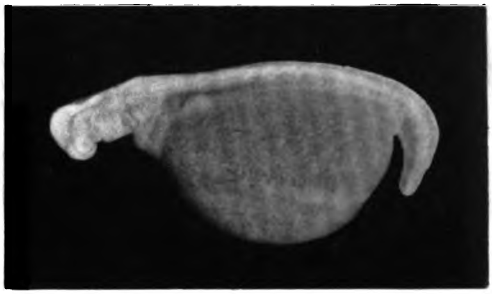

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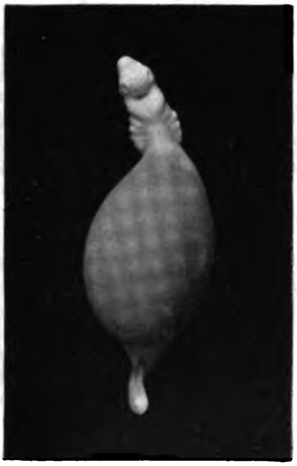

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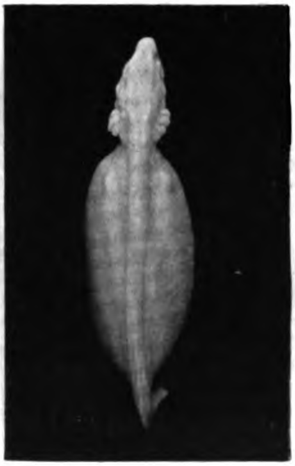

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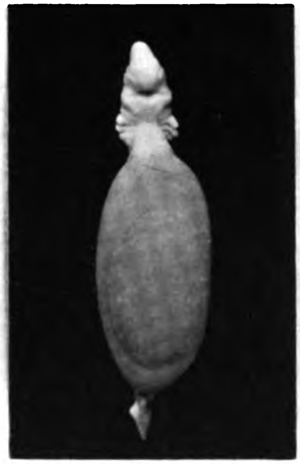

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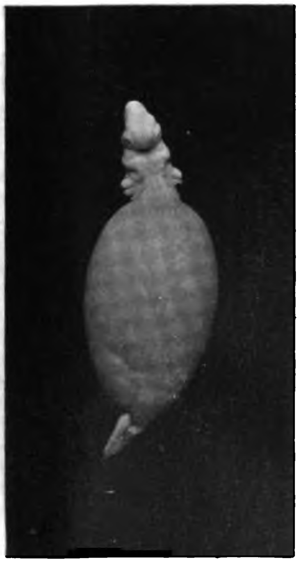

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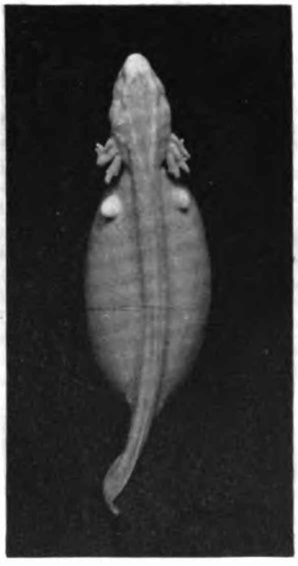

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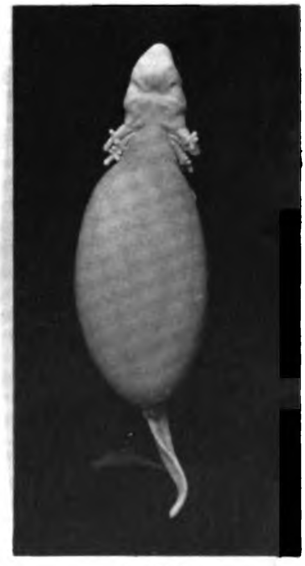

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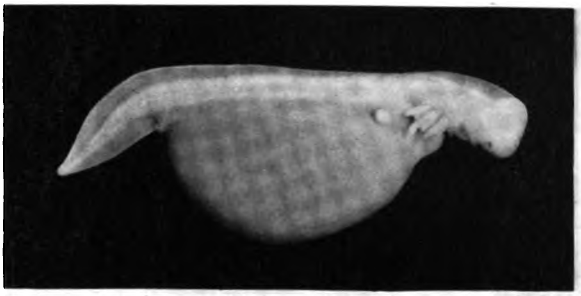
PLATE 8

EXPLANATION OF PLATES

(Cryptobranchus allegheniensis)

Bichromate-acetic-formalin fixation except for figures 260 and 261 which were made from living specimens anaesthetized with chloretone. × 4.

258 Stage 22. Lateral view.
259 Stage 22.5. Lateral view.
260 Stage 23. Dorsal view of an embryo ready to hatch.
261 Stage 23. Lateral (slightly ventral) view of a newly-hatched larva.



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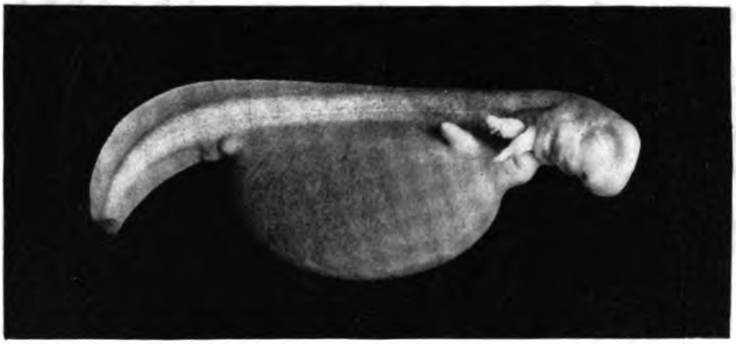

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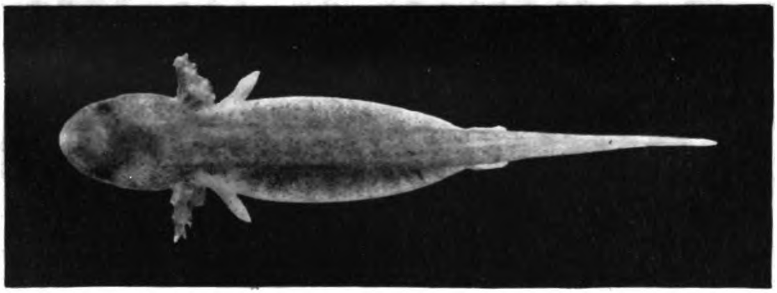

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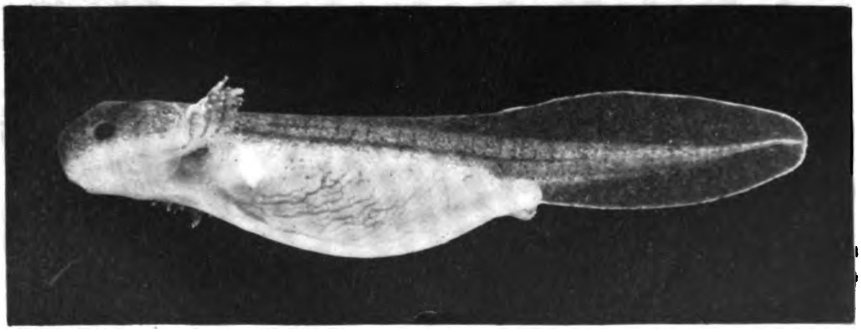

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PLATE 9

EXPLANATION OF FIGURES

(Cryptobranchus allegheniensis)

All the figures are natural size.

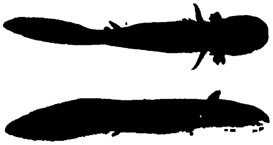
262 Living larvae reared in the laboratory, anaesthetized with chloretone and photographed about five weeks after hatching.

263. Larva reared in the laboratory, killed in bichromate-acetic-formalin about two months after hatching and preserved in formalin. The specimen is slightly shortened through the action of the fixing fluid.

264 and 265. Two views of a larva reared in the laboratory, anaesthetized with chloretone and photographed about ten weeks after hatching.

266. Larva reared in the laboratory, anaesthetized with chloretone and photographed about six months after hatching.

267. Year-old larva captured and fixed in formalin August 27, 1909; a few weeks later it was transferred to alcohol, in which it remained nearly a year before being photographed.



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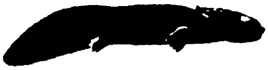

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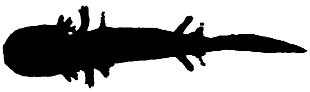

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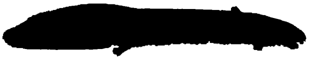

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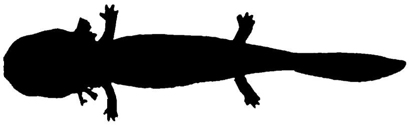

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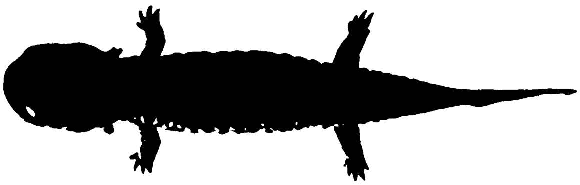


267

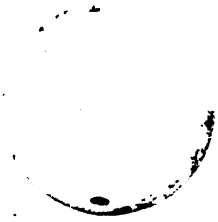
PLATE 10

EXPLANATION OF FIGURES

(Necturus maculosus)

The figures are intended to show in particular the late history of the blastopore. Figures 268 to 276 inclusive were drawn from the living eggs by Prof. Bashford Dean; figures 277 to 279 are from preserved material, and were drawn under the direction of the author by Miss Mabel L. Hedge.

Necturus maculosus

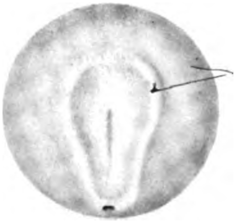


268

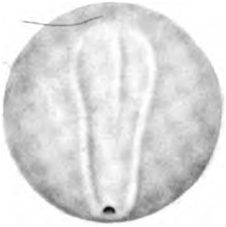
269

270

271

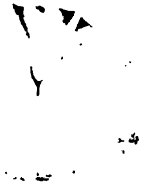


272



273

274



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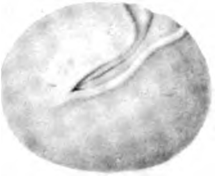
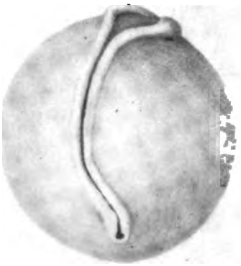
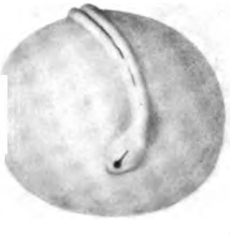


276

277



278



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